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EFFECTS OF PARA AMINOBENZOIC ACID AND THIOURACIL ON THYROID FUNCTION AND RESISTANCE TO LOW PRESSURES[1]

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MUCH ATTENTION has been focussed recently on the effects of thiourea and thiouracil on thyroid structure and function. These drugs have been found to induce the formation of an enlarged hyperplastic thyroid gland in the mammal, bird, amphibian and fish. This state is associated with a condition of functional hypothyroidism due to the interference by the drugs with the production of normal thyroid principle. Recently it has been reported that rats treated with these drugs display an increased resistance to lowered barometric pressures (Gordon, Goldsmith and Charipper, 1944a; Leblond, 1944; Hughes and Astwood, 1944) which results, at least partly, from the ability of these agents to interfere with thyroid function.

The aminobenzoic acids also have been found to produce a goitrogenic effect in rats (the Mackenzies, 1943). This is of considerable interest since para aminobenzoic acid (PABA), a naturally occurring food factor, is the most effective of the three (Astwood, 1943). The effect of PABA, however, is less pronounced than that of thiourea or thiouracil. Since the studies with PABA have not been extensive it was decided to make a more detailed examination of the action of this substance on thyroid function and to compare these effects with those exerted by thiouracil. In addition, information on the comparative effects of these agents on the formed elements of the blood will also be presented since this, along with the body weight records, serves as

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an indication of the relative toxicity of these substances. A preliminary report on some phases of this work has already been published (Gordon, Goldsmith and Charipper, 1944b).

MATERIALS AND METHODS

Adult male rats weighing 220–280 grams were employed in all the experiments. The animals were fed a laboratory ration containing either 0.2% thiouracil[2] or 3.0% PABA[3] for periods varying from 19 to 45 days. After 38 days of treatment, total red, white cell counts and differentials were made in the usual way from tail blood. Hemoglobin was estimated with a Fisher electro-hemometer. Reticulocyte percentages were determined from wet smears stained with brilliant cresyl blue. The diets of several groups fed the PABA were supplemented with 0.2% thiamine hydrochloride. After

TABLE I. EFFECTS OF PABA AND THIOURACIL ON BODY GROWTH, THYROID WEIGHT OXYGEN CONSUMPTION AND RESISTANCE TO REDUCED BAROMETRIC PRESSURES (190 MM HG) OF ADULT MALE RATS. MEAN FIGURES ARE GIVEN WITH THEIR STANDARD ERRORS.

	Paba						Thiouracil				
Days treat.	No. anim.	Wt. gain (gm.)	Thyr. wt. (mg.)	O_2 Cons. (cc/cm²/hr.)	Survival time (mins.)		No. anim.	Wt. gain (gm.)	Thyr. wt. (mg.)	O_2 Cons. (cc/cm²/hr.)	Survival time (mins.)
19–22	12	34	26.2 ± 1.2	0.75 ± 0.03	61.2 ± 8.3(5*)		12	19	53.5 ± 2.0	0.71 ± 0.03	>120(11*)
26–30	13	46	37.0 ± 2.0	0.73 ± 0.02	83.8 ± 6.3(7*)		12	26	67.8 ± 3.2	0.65 ± 0.015	>120(12*)
40–45	12	52	41.2 ± 3.8	0.69 ± 0.02	78.3 ± 6.1(6*)		12	27	74.3 ± 3.1	0.67 ± 0.02	>120(12*)
			Controls								
0	36	(28)† (40) (50)	17.2 ± 0.32	0.97 ± 0.01	35.1 ± 3.2(4*						

* Number of animals surviving the 2 hour exposure to 190 mm. Hg.
† Weight gains 20, 28, and 42 days after start of the experiment.

the 3 periods of treatment indicated in Table I, the oxygen consumptions were measured according to the method of Tainter and Rytand (1932). The rats were then placed into a large cylindrical low pressure chamber. The pressure was reduced over the course of 20 minutes to a value of 190 mm. Hg (34,000 feet) at which point it was maintained for 2 hours. During this period the time for complete cessation of breathing was recorded. After 2 hours the pressure was further reduced over the course of an additional hour to a level of 141 mm. Hg (40,000 feet) and the times of death of the remaining animals noted. At the end of each experiment all rats were examined for the extent of lung hemorrhage (Lawson, Carnes and Thienes, 1943) and eye cataract (Bellows and Nelson, 1944). The thyroid and adrenal glands were carefully dissected and weighed to the nearest milligram on a torsion balance. Thyroid and pituitary glands from representative animals in each series were fixed in Bouin's, Zenker-formol or Champy's solutions and prepared for microscopical examination.

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[2] Thanks are extended to Drs. B. W. Carey and S. Hardy of the Lederle Laboratories, Inc., for a supply of thiouracil.
[3] Dr. Hans Molitor, Merck and Co., kindly supplied the PABA.

RESULTS

Effects on thyroid, pituitary and adrenal.—The data of Table I indicate that both thiouracil and PABA caused a significant increase in the size of the thyroid gland. The greater activity of thiouracil was reflected in the histological appearance of the thyroid (compare Figs. 1 and 2) and pituitary glands (compare Figs. 3 and 4) of the rats receiving the drug. None of the treatments was found to affect significantly the adrenal weights.

Oxygen consumption.—Table I shows that both thiouracil and PABA reduced the oxygen consumption approximately 25–35% below normal by the 3rd week of treatment. Longer periods of treatment (i.e., up to 45 days) appeared to cause no further significant lowering of the metabolic rate.

TABLE II. EFFECTS OF 38 DAYS OF TREATMENT WITH PABA AND THIOURACIL ON FORMED ELEMENTS OF THE BLOOD AND HEMOGLOBIN IN THE RAT. MEAN FIGURES ARE GIVEN WITH THEIR STANDARD ERRORS.

Treatment	No. anim.	RBC (mill./cu. mm.)	Hb (gm./100 cc.)	Retics. (%)	WBC (thous./ cu. mm.)	Differential (%)			
						Neutroph.	Eosin.	Lymph.	Monoc.
Paba	13	8.4±0.17	16.4±0.23	1.6±0.21	20.7±1.2	20.0±0.81	2.1±0.28	76.0±0.90	2.0±0.23
Thiouracil	12	7.4±0.19	14.5±0.16	1.9±0.15	19.0±1.7	5.5±0.31	1.6±0.13	89.3±0.52	3.2±0.39
Controls	20	8.5±0.13	15.2±0.27	1.2±0.22	22.3±1.8	22.2±0.73	2.3±0.18	73.8±0.90	1.5±0.21

Resistance to lowered barometric pressures.—Of the 36 untreated male rats subjected to the pressures of 190 mm. Hg only 4 survived the intial 2 hour period. On the other hand, 18 of the 37 PABA-treated rats and 35 of the 36 thiouracil-fed rats tolerated the 2 hour period of exposure. Table I also shows that the PABA-treated rats which failed to survive during the first 2 hours nevertheless lived for a longer period of time than the untreated controls. The addition of 0.2% thiamine hydrochloride to the diets of rats fed PABA afforded no extra protection to the lowered pressures. Neither of the drugs appeared to prevent the development of lung hemorrhage or lens cataract which occurred in approximately 75% of both the control and experimental animals.

Toxic effects.—Table I reveals that the rats fed the thiouracil failed to grow as rapidly as the controls given ordinary laboratory ration. On the other hand, the weight gains of the rats fed PABA compared favorably with those experienced by the untreated controls.

Table II shows that the administration of thiouracil for 38 days resulted in granulocytopenia, lymphocytosis, and reductions in total red cell counts and hemoglobin concentrations of the blood. PABA, given for the same period of time, caused no significant alterations in the blood picture of the rat.

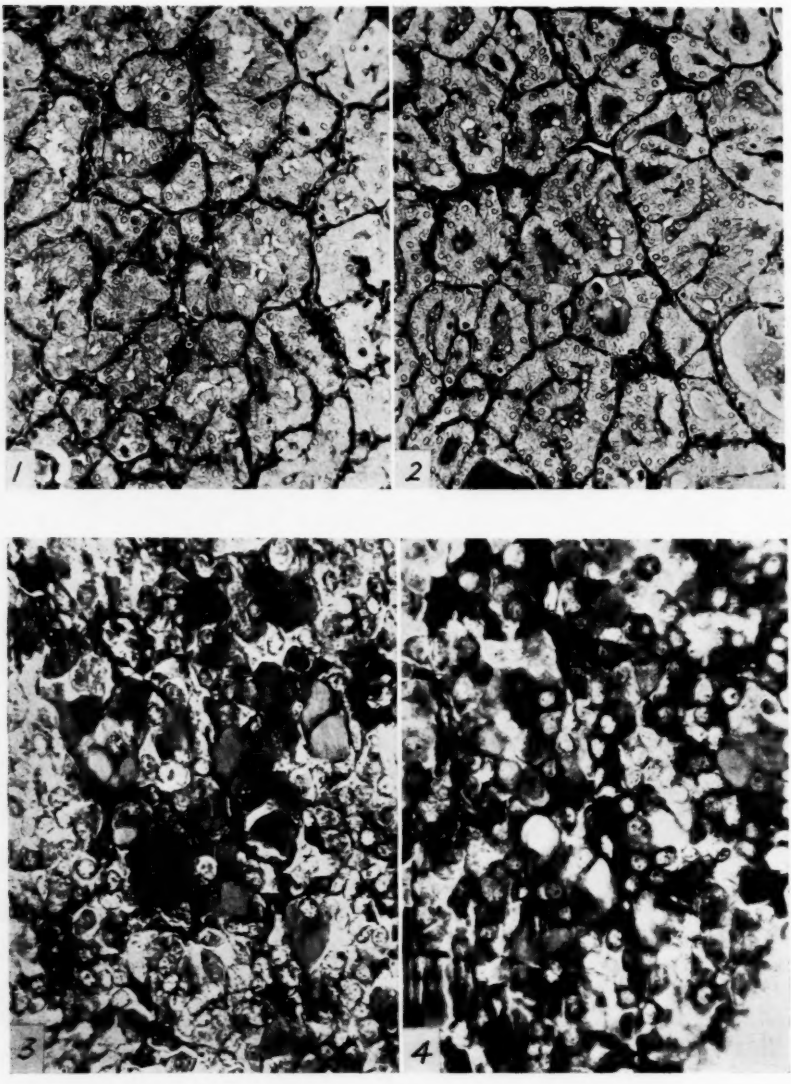

FIG. 1. Thyroid of rat fed 0.2% thiouracil for 40 days. Note extremely active condition (e.g., enlarged irregular follicles, tall columnar epithelium, absence of colloid, intense hyperemia). Zenker-formol fixation; Masson's trichrome stain. ×240.

FIG. 2. Thyroid of rat fed 3.0% PABA for 40 days. Although gland is considerably activated, the extent of activation is not as great as that seen in Fig. 1. Zenker-formol fixation; Masson's trichrome stain. ×240.

FIG. 3. Anterior pituitary of rat fed 0.2% thiouracil for 40 days. Many enlarged

DISCUSSION

The present experiments confirm the previous findings by the Mackenzies (1943) and Astwood (1943) that thiourea compounds are more effective goitrogenic agents than the aminobenzoic acids. This is seen not only from the larger-sized goiters occurring in the thiouracil-treated rats, but also in the greater numbers of "thyroidectomy" cells and more pronounced basophilic condition of their anterior hypophyses.

It is well known that removal of the thyroid gland is beneficial (Barach, Eckman and Molomut, 1943) and administration of thyroid or anterior pituitary substance detrimental (Houssay and Rietti, 1932; Campbell, 1935) to animals exposed to anoxic conditions. It thus seems possible that the protection given by thiourea (Gordon, Goldsmith and Charipper, 1944a; Leblond, 1944) or thiouracil (Hughes and Astwood, 1944; Goldsmith, Gordon and Charipper, 1945) to rats subjected to reduced pressures is due to the interference by these drugs with the formation of thyroid principle. Despite the fact that PABA in these experiments was given in approximately 8 times the molar strength of thiouracil, the latter still afforded considerably greater protection to animals placed under low pressures. The explanation for this difference is not clear. Depression in basal oxygen consumption does not appear to be the only factor concerned since both substances reduced the oxygen intake to approximately the same extent. It may be true, as Hughes and Astwood (1944) have suggested, that such drugs may operate by enabling the animals to utilize oxygen more efficiently or by making an anaerobic source of energy available. If this is the case then thiouracil must effect these changes more readily than PABA.

Even though PABA was employed in much greater concentration than thiouracil, it had no effect on body growth or on the formed elements of the blood of adult rats. Thiouracil, on the other hand, inhibited the body weight gains noted in both normal and surgically-thyroidectomized adult rats (Gordon, Goldsmith and Charipper, 1945). It also induced a slight anemia, granulocytopenia, and lymphocytosis. These effects on the blood picture are similar to those obtained with thiourea (Goldsmith *et al.*, 1944). Anemia with thiouracil has also been found in rats by Hughes (1944) and Williams *et al.*, (1944). The latter workers also reported leucopenia in thiouracil-fed rats.

It should be noted that the granulocytopenic state observed with

vacuolated basophilic elements ("thyroidectomy" cells) are seen; eosinophilic cells are almost completely absent. Champy's fixation; Mallory-azan stain. ×470.

FIG. 4. Anterior pituitary of rat fed 3.0% PABA for 40 days. Several "thyroidectomy" cells are apparent; number and size are not as great as seen in Fig. 3. Well-defined eosinophiles can still be detected. Champy's fixation; Mallory-azan stain. ×470.

thiourea and thiouracil in rats is a chronic one which develops early, is maintained for as long as 6 months, and is not necessarily associated with a decrease in total white cell count. Thus it differs from the condition in the thyrotoxic human treated with thiouracil in which granulocytopenia may develop suddenly, is almost always associated with marked leucopenia, and may prove fatal. Berman (1945) has recently reported success in the treatment of thyrotoxicosis in humans with PABA. Although here, too, large amounts were administered (e.g., 1.5–2.0 grams PABA parenterally 6 times a week) no untoward symptoms were noted in any of the patients for periods of treatment as long as 9 months.

The possibility of the use of relatively non-toxic substances like PABA in increasing the aviator's tolerance to anoxia has been considered. This, however, would entail the administration of great quantities of the material for an impractical period of time. Moreover a goitrous state (even though less marked than with thiouracil) which is generally associated with a reduction in general efficiency due to the hormone lack, would certainly predicate against the use of such materials. Although the use of drugs, food factors, and hormones in increasing the tolerance and efficiency of the human at high altitudes still remains an interesting possibility, many more detailed studies must be conducted before the practicability of such experiments can be admitted.

SUMMARY

Both thiouracil and PABA (0.2% and 3.0% of the food respectively) administered for 19–45 days caused marked thyroid hyperplasia, basophilia and appearance of "thyroidectomy" cells in the anterior hypophysis, and increased resistance of rats to lowered barometric pressures (190 mm. Hg). These effects, however, were more pronounced with thiouracil than with PABA.

Both drugs reduced the B.M.R. to approximately the same extent; neither altered the resistance of the rats to the degree of lung hemorrhage or extent of eye cataract encountered at the reduced pressures.

Thiouracil inhibited normal weight gains and, after 38 days, induced slight anemia and granulocytopenia. PABA, on the other hand, did not slow body growth and exerted no effect on the blood picture.

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EFFECTS OF THYROTROPHIN ON METABOLISM OF THIOURACIL-TREATED RATS

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MUCH EVIDENCE has been accumulating that thiourea and thiourea derivatives depress the ability of the thyroid gland to secrete thyroid hormone. Thus, the ability of the gland to synthesize organic iodine has been found lowered (Franklin, Chaikoff and Lerner, 1944), the level of circulating organic iodine decreased (Salter and McKay, 1944) and the Basal Metabolic Rate (B.M.R.) depressed (Astwood, Sullivan, Bissell and Tyslowitz, 1943; MacKenzie and MacKenzie, 1943).

Because of the importance of the anterior pituitary thyrotrophin in controlling thyroid secretion, it was thought worthwhile to determine the metabolic response of the thiouracil-treated animal to this hormone. Jandorf and Williams (1944) have reported tissue metabolism studies on such animals, but did not include whole-animal changes. Williams and Bissell (1943) reported that thyrotrophin did not raise the B.M.R. of a patient also receiving thiouracil, but no standard response to the thyrotrophin was presented.

METHODS

Adult albino rats weighing 200–300 gm. were used in these experiments. The animals were bred in this laboratory from the Sprague-Dawley strain.

The resting metabolic rate of each rat was determined in a Benedict closed-circuit multiple-chamber apparatus which was tested frequently for tightness (Benedict, 1930). The usual procedure of imposing a 24-hour fast before the determination was changed so as to allow successive determinations without detriment to the animals. This was accomplished by restricting the rats' feeding period to 5 hours in the afternoon or evening. Sixteen hours after the removal of the food containers, the metabolic rate of the selected animals was run. By this means, a 3-hour period was available for the study each day, without any interference with the next feeding period of the animals. Justification of this procedure has been thoroughly worked out (Barker unpublished). All metabolic rate figures are shown as cc. oxygen consumed per 100 grams of body weight per hour in order to avoid the many assumptions involved in computing the B.M.R. as Calories per square meter of body surface per hour.

Thiouracil[1] was at first administered to the rats in the drinking water.

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[1] Thiouracil was kindly furnished by Dr. Stanton M. Hardy of the Lederle Laboratories.

Despite reports that it could be dissolved to a concentration of 0.1 per cent, we consistently observed crystals deposited in the glass water containers from any solution stronger than 0.05 per cent. Our rats drank about 25 cc. per day, resulting in a dosage of about 50 mg. per kg. body weight. At this level, effects on the B.M.R. were sufficiently inconsistent as to warrant increasing the dosage. Accordingly, the drug was added to the Purina dog checkers used in group-up form. Repeated testing indicated that thiouracil to 0.5 per cent by weight of food should be provided. This gave a dose of about 200–250 mg. per kg. body weight.

After a few preliminary trials, thyrotrophin[2] was given in a single subcutaneous injection, 40 Junkmann-Schoeller guinea-pig units per 100 gm. body weight. It was found that the peak of the metabolic response was usually obtained 24 hours following the single injection and remained at this level for approximately another 24 hours. A few runs were made using 20u. per 100 gm. body weight on each of four consecutive days. The height of the elevated oxygen consumption was reached 24 hours after the last injection. Although this latter procedure produced a somewhat greater elevation of B.M.R. and a slower return to normal, the comparative results were the same as with one larger dose.

Desiccated thyroid substance[3] was administered by stomach tube. Each rat was given 100 mg. per 100 gm. body weight as a 10 per cent suspension in water in a single dose. Here the peak of the response was usually not reached until the second or third day. The single dose technique is preferred as furnishing more consistent results than repeated administrations.

RESULTS

Table 1 summarizes the effects of 200–250 mg. thiouracil per kg. body weight on the basal metabolism of 29 rats, both males and females.

TABLE 1. EFFECT OF THIOURACIL ON BASAL METABOLIC RATE OF RATS

Animals		Normal	Thiouracil				
Sex	No.	Basal[1]	Basal[1] 14 d.	Basal[1] 21 d.	Total Days	Average Basal[1]	Day Reached
M	16	99.1	90.4 −8.8%	87.3 −11.9%	104	86.3 −12.9%	26
F	13	101.1	92.3 − 8.7%	91.5 − 9.5%	82	89.6 −11.4	22
Both	29	100.0	91.3 − 8.7%	89.2 −10.8%	94	87.7 −12.3%	24

[1] cc. oxygen per 100 gm. body weight per hour.

[2] Antuitrin-thyrotrophic was generously provided by Dr. J. J. Pfiffner of Parke, Davis and Co.

[3] The desiccated thyroid was courteously supplied by Dr. I. J. Klingaman of the Cudahy Packing Co.

observations. At this time, the average basal was 8.7 per cent below the pre-treatment level. An additional 7 days changed this figure to 10.8 per cent. Despite the fact that some animals were maintained on thiouracil for 100 days more, there was little further metabolic depression found. The average oxygen consumption levelled off by the 24th day, at 87.7 cc. per 100 gm. body weight per hour, 12.3 per cent below the starting average, and was maintained for an average total of 94 days.

All tables list averages separately for males and females. It will be noted that there was a constant tendency for the females to show

TABLE 2. EFFECT OF SINGLE DOSE OF THYROTROPHIN ON BASAL METABOLIC RATE OF RATS

Animals			Oxygen Consumption[1]		
Condition	Sex	No.	Control	Th. T.[2]	Increase
Normal	M	8	99.4	123.7	24.3 (+24.4%)
Normal	F	7	98.9	123.9	25.0 (+25.3%)
Normal	Both	15	99.2	123.8	24.6 (+24.8%)
Thiouracil 14–21 d.	M	8	86.7	101.3	14.6 (+16.8%)
	F	8	91.1	104.5	13.4 (+14.7%)
	Both	16	88.9	102.9	14.0 (+15.7%)
Thiouracil 24–55 d.	M	8	86.4	93.1	6.7 (+7.8%)
	F	8	92.2	96.7	4.5 (+4.9%)
	Both	16	89.3	94.9	5.6 (+6.3%)

[1] Oxygen consumption, cc. oxygen per 100 gm. body weight per hour.
[2] Thyrotrophin, 40 u. per 100 gm. body weight.

a less marked response to thiouracil than the males. In no instance, however, was the difference sufficiently marked to alter the conclusions.

Table 2 shows that 40u. thyrotrophin per 100 gm. body weight in one injection produced an average rise of 24.8 per cent in 15 normal rats. Eight of these animals were males and 7 females; their responses were nearly identical, and were averaged together. Sixteen thiouracil-treated animals were each subjected to two thyrotrophin injections, the first between the 14th and 21st days on thiouracil, and the second between the 24th and 55th. As can be seen from Table 2, the suppression of thyroid activity was sufficient by the earlier time to decrease the response to thyrotrophin to 14.0 cc. oxygen, or 15.7 per cent above the 11.4 per cent depressed basal. Although these 16 animals showed no further depression of basal after 21 days on thiouracil, the stimulating effect of thyrotrophin was markedly less, being only 5.6 cc. oxygen, or an increase of 6.3 per cent above the basal average of 89.3 cc. oxygen.

In an effort to produce an even more striking difference, half of the previous dose of thyrotrophin was injected on each of four suc-

cessive days (Table 3). Four normals responded with an average increase of 32.3 per cent above their basals, while 9 rats on thiouracil for a duration of 30–66 days responded with 5.9 cc. oxygen increase, or +6.7 per cent above the 12.3 per cent depressed basal. The difference between these responses is thus somewhat greater than that with the single dose after prolonged thiouracil administration.

Other workers (Dempsey and Astwood, 1943; Reineke, Mixner and Turner, 1945; Meyer and Ransom, 1945) have reported a good

TABLE 3. EFFECT OF REPEATED THYROTROPHIN ON BASAL METABOLIC RATE OF RATS

Animals			Oxygen Consumption[1]		
Condition	Sex	No.	Control	Th. T.[2]	Increase
Normal	M	4	95.7	126.6	30.9 (+32.3%)
Thiouracil 30–66 d.	M	4	86.3	89.8	3.5 (+4.1%)
	F	5	88.9	96.7	7.8 (+8.8%)
	Both	9	87.7	93.6	5.9 (+6.7%)

[1] Oxygen consumption, cc. oxygen per 100 gm. body weight per hour.
[2] Thyrotrophin, 20 u. per 100 gm. body weight injected once a day for 4 consecutive days.

TABLE 4. EFFECT OF DESICCATED THYROID ON BASAL METABOLIC RATE OF RATS

Animals			Oxygen Consumption[1]		
Condition	Sex	No.	Control	Th.[2]	Increase
Normal	M	14	101.3	136.1	34.8 (+34.4%)
	F	4	98.0	141.1	43.1 (+44.0%)
	Both	18	100.6	137.2	36.6 (+36.4%)
Thiouracil	M	11	84.7	117.5	32.8 (+38.7%)
	F	8	92.1	121.4	29.3 (+31.8%)
	Both	19	87.8	119.1	31.3 (+35.6%)

[1] Oxygen consumption, cc. oxygen per 100 gm. body weight per hour.
[2] Desiccated thyroid, 100 mg. per 100 gm. body weight.

response of thiourea- and thiouracil-treated rats to continued administration of desiccated thyroid, indicating that the extra-thryoid tissues could still respond to thyroid hormone itself. Table 4 shows further data bearing on this point. Although the animals on thiouracil gave only 31.3 cc. oxygen increase as compared to 36.6 in the controls, the percentage rises were nearly the same, +35.6 and +36.4 per cent respectively. It is difficult to decide whether one should conclude, on the basis of the actual oxygen consumption figures, that the tissues were able to respond only to 85 per cent of the normal extent, or, on the basis of percentages of the basal metabolic rates, that the responses were practically identical. One can conceive of the possibility that the tissues exhibiting a depressed metabolism would be capable of responding only proportionately to the basal, although this situation

is a considerable contrast to the thyroid sensitivity of the hypothyroid human (Winkler, Criscuolo and Lavietes, 1943).

DISCUSSION

These results show that the animal with basal metabolic rate depressed by thiouracil gives a much smaller metabolic response to injected thyrotrophin than the normal. Since the response to administered thyroid material is only slightly altered, the metabolic defect due to thiouracil seems located in the thyroid gland rather than in the extra-thyroid tissues.

This is further support of the concept that the thiourea derivatives interfere with thyroid hormone formation and secretion. Despite such a block, the gland appears able to show considerable hypertrophy, hyperplasia and hyperemia, presumably in response to increased endogenous thyrotrophin secretion as the thyroid hormone level falls. In fact, the changes in the thyroid can be prevented by hypophysectomy (Astwood and Bissell, 1944) or by administration of thyroxin (MacKenzie and MacKenzie, 1943; Astwood, Sullivan, Bissell and Tyslowitz, 1943).

Jandorf and Williams (1944) have shown that thyroid glands extirpated from thiouracil-treated rats exhibited a greatly increased oxygen consumption, in contrast to the normal metabolic rates of liver and diaphragm of the same animals. Stimulation of thyroid metabolism by thyrotrophin injections, previously demonstrated in normal rats by Canzanelli and Rapport (1937) and by Belasco and Murlin (1941), was also found by Jandorf and Williams to occur despite thiouracil treatment. Liver and diaphragm responded well following thyrotrophin in the animals with normal thyroid function, but gave no reaction in the thiouracil-depressed rats.

Whether the residual effects of thyrotrophin shown in our animals long treated with thiouracil truly represent some remaining secretory ability of the thyroids, or simply side effects of the preparation employed cannot finally be decided from these data. However, since the principal contaminent is gonadotrophic in nature, it is considered improbable that it would be responsible for any considerable elevation of metabolism in one or two days. Following the four-day regime, autopsy revealed considerable growth and hyperemia of gonads and accessory sex structures in both males and females. These changes must be considered as a part of the response under the repeated dosage routine; in the animals autopsied after a single dose, such side effects were much less marked.

Some comment seems appropriate concerning the basals before thyrotrophin. Each of these animals was thoroughly trained and at least two basal determinations obtained before administration of the drug was begun. Therefore, considerable confidence is felt in the results. The maximum depression in B.M.R. ever obtained was 23 per cent; inasmuch as the average of all thiouracil basals was 87.7 cc.

oxygen per 100 gm. of body weight per hour, or 12.3 per cent below the untreated level of 100.0, it is apparent that many animals did not show a depression at all comparable to the 23 per cent maximum. Six animals were encountered which showed changes of −1.2, −2.9, −4.0, −4.0, −4.2 and −4.6 per cent, respectively. That is, 6 of the total of 29 animals gave an average decrease of only 3.5 per cent from their original basal levels. The remaining 23 averaged −14.4 per cent, ranging from −6.5 to −22.7 per cent.

Many of these changes following thiouracil are considerably less than values previously reported in the literature. Movement will not account for the differences; all animals were kept under observation, and any excess movement was considered adequate justification for terminating that particular experiment without even calculating the data. Although these animals received the drug only during the feeding time of 5 hours, it is felt that absorption from the gastrointestinal tract beyond this time, as well as possible storage in the thyroid and other tissues (Williams, Kay and Jandorf, 1944), would make sufficient thiouracil available for a continued depression of the thyroid. No consistent difference in thyrotrophin effect was obtained between animals with a marked lowering of B.M.R. and those showing less response. Furthermore, histological examination of thyroids of animals typical of each group revealed the same degree of colloid exhaustion and cellular alterations in each case.

SUMMARY

Thiouracil was administered to 29 albino rats at a dosage of 200–250 mg. per kg. body weight per day. The B.M.R. was depressed an average of 12 per cent by the 24th day, and showed no further change for 94 days. An amount of thyrotrophin which caused an increase of 24.8 per cent in the B.M.R. of 15 normal animals gave only +15.7 per cent when injected into 16 rats treated with thiouracil for 14–21 days. When these same 16 animals had continued on the thiouracil regime for an additional period of 10–40 days, the response to thyrotrophin was only +6.3 per cent. A somewhat greater difference was obtained after repeated injections of thyrotrophin: +32.3 per cent for the normals, and +6.7 per cent for those on thiouracil. The actual increases in oxygen consumption were even less, since these percentages were calculated from the lowered basals. Administration of desiccated thyroid material produced an essentially normal elevation of B.M.R. in thiouracil-treated rats. It is concluded, therefore, that this evidence gives further support to the concept that thiouracil action is principally exerted on the production of thyroid hormone.

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THE HORMONAL INDUCTION OF MATING RESPONSES IN THE SPAYED-ADRENALECTOMIZED FEMALE RAT

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THE CONSISTENT induction of mating behavior in the spayed females of several species is dependent upon the injection of estrogen and progesterone in proper sequence (guinea pig, Dempsey, Hertz and Young, 1936; rat, Boling and Blandau, 1939; mouse, Ring, 1944). It is assumed that this is the estrus-mechanism operating in normal females, at least in these species, the progesterone being secreted by luteinizing granulosa cells in the preovulatory follicle. For a review of the literature in this connection see Young (1941).

If the normal induction of estrus depends upon the synergistic action of follicular estrogen and progesterone, it is necessary to explain the mechanism by which heat (usually low grade) irregularly follows injections into spayed animals of relatively large amounts of estrogen alone. One possible interpretation is that injected estrogen stimulates the production and release of progesterone or a progesterone-like steroid from an extra-ovarian source.

In view of the close molecular similarity between progesterone and the adrenal cortical steroids, especially desoxycorticosterone, and the capacity of cortical extracts as well as crystalline cortical hormones to substitute for progesterone in the induction of mating in spayed guinea pigs (van Heuverswyn, Collins, Williams and Gardner, 1939; Torstveit and Mellish, 1941), it seems logical to look to the adrenal cortex as the postulated extra-ovarian source of progesterone activity.

The present experiments have been devised to test the hypothesis that sexual receptivity (heat) may be induced in spayed female rats by injections of estrogen alone only because of the presence of progesterone or progestimimetic substances derived from the adrenal cortex.

MATERIALS AND METHODS

Forty-two albino female rats of the Wistar strain ranging in age from 3 to 8 months were used in these experiments. Thirty of the animals were spayed and, after a suitable time had elapsed, injected subcutaneously with graded concentrations in sesame oil of estradiol benzoate alone or followed 72 hours

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later by progesterone.[1] Subsequently the animals were adrenalectomized bilaterally in a one-stage operation and thereafter maintained with the aid of a solution of 1.5 per cent NaCl and 0.5 per cent $NaHCO_3$ administered *ad libitum* as drinking water. The diet was a commercial fox food in pellet form supplemented once or twice a week with carrots and greens. The remaining 12 animals were spayed and adrenalectomized in a single operation and maintained in the same way.

The animals were permitted a recovery period of at least a week after adrenalectomy before injections were begun. Three animals failed to survive for this period. The average survival time of those animals which showed surgical recovery but subsequently died in adrenal insufficiency was 28 days, range 13 to 58 days. The rats were allowed to rest between injections until diestrous vaginal smears were obtained, a period of 1 to 2 weeks. At death autopsies were performed, and a search was made for regenerated adrenal tissue or hypertrophied accessory cortical tissue at the adrenal site.

The spayed-adrenalectomized rats were injected in the same manner as the spayed animals with estrogen alone or followed at 72 hours by progesterone. Heat was detected by using the manual stimulation method of Blandau, Boling and Young (1941), occasionally verified by placing the females with active males. Observations were made during the day at 1 or 2 hour intervals beginning 24 hours after the estrogen injection. At night the injected females were caged with males and a search for copulation plugs made the following morning. Boling and Blandau (1939) found the average latent period for the action of estrogen in inducing heat in spayed rats to be about 52 hours, with the majority of animals coming into heat between 50 and 60 hours after the estrogen injection. Preliminary results confirmed this time, consequently observations were made at hourly intervals during the period from 48 to 60 hours after the estradiol injection.

With the exception of the first experiment, those animals which had not come into heat during the 72 hours following the estradiol injection were injected with progesterone and observed for 8 more hours at hourly intervals. Those still not in heat were placed with males and examined the following morning for copulation plugs.

To test the possibility that some animals might have a longer latent period than 72 hours, one group of spayed rats and one group of spayed-adrenalectomized rats were observed for 96 hours after the injection of 100 R.U. of estradiol benzoate. These animals did not receive subsequent progesterone treatment.

RESULTS

Induction of Heat in Spayed Rats

Table 1 presents the results of the injections of estradiol benzoate in inducing heat in spayed female rats. These data afford qualitative confirmation of the work of Boling and Blandau (1939), although the percentage of matings obtained by them with injections of 100 R.U. of estradiol benzoate (18 of 20 injections followed by heat) is considerably higher than similar groups in the present experiments. With

[1] Progynon-B and Proluton kindly furnished by Dr. E. Schwenk of the Schering Corporation.

smaller amounts of estrogen the results in the two series are quite comparable.

In the group of 9 animals observed for 96 hours one rat came into heat at 76 hours, whereas the others had latent periods ranging from less than 50 to less than 69 hours. Among the animals receiving 100 R.U. of estrogen, the lower percentage mating in the group observed for 96 hours has no significance. On the other hand the fact that only one animal had a latent period greater than 72 hours suggests that

TABLE 1. INDUCTION OF HEAT IN SPAYED FEMALE RATS BY INJECTIONS OF ESTRADIOL BENZOATE

Amount of estradiol benzoate	Duration of observations	Number of rats injected	Number of injected rats in heat	% injected rats in heat
R.U.	hours			
10	72	32	8	25
25	72	22	4	18
50	72	19	5	26
100	72	13	7	54
100	96	9	4	44

TABLE 2. INDUCTION OF HEAT IN SPAYED FEMALE RATS BY INJECTIONS OF PROGESTERONE (0.5 MG.) 72 HOURS AFTER INJECTION OF ESTRADIOL BENZOATE

Amount of estradiol benzoate	Number of rats injected	Number of injected rats in heat	% injected rats in heat
R.U.			
10	14	14	100
25	10	10	100
50	10	10	100
100	6	6	100

no large number of animals would have come into heat had the observations been of longer duration.

The heat displayed by spayed rats following injections of even maximal quantities of estrogen alone is of low intensity as compared with the normal estrous female and spayed females receiving estrogen and progesterone in sequence.

In all but one experiment, those females which did not come into heat during the 72 hours following the injection of estradiol benzoate were injected with 0.5 mg. of progesterone. Table 2 summarizes the results of these injections. Strong mating responses could be elicited from all animals after latent periods of from 2 to 6 hours, the average being 4 hours after the progesterone injection.

Induction of Heat in Spayed-adrenalectomized Rats

Data concerning the response of spayed-adrenalectomized rats to injections of estradiol benzoate are summarized in Table 3. The percentages of animals coming into heat following estrogen injection are

substantially reduced by adrenalectomy, although mating was not eliminated. It will be seen below, however, that in most instances the animals responding to estrogen alone in this series proved at autopsy to have persistent cortical tissue. The mating reactions displayed were not unlike those following injections of estrogen into spayed rats with adrenals intact.

In the case of those animals observed for 96 hours, the animals which came into heat did so before the third day, the average latent period being about 52 hours. Thus the two 100 R.U. groups may be

TABLE 3. INDUCTION OF HEAT IN SPAYED-ADRENALECTOMIZED FEMALE RATS BY INJECTIONS OF ESTRADIOL BENZOATE

Amount of estradiol benzoate	Duration of observations	Number of rats injected	Number of injected rats in heat	% injected rats in heat
R.U.	hours			
10	72	19	2	11
25	72	16	1	6
50	72	24	4	17
100	72	17	3	18
100	96	13	2	15

TABLE 4.—INDUCTION OF HEAT IN SPAYED-ADRENALECTOMIZED FEMALE RATS BY INJECTIONS OF PROGESTERONE 72 HOURS AFTER INJECTION OF ESTRADIOL BENZOATE

Amount of estradiol benzoate	Amount of progesterone	Number of rats injected	Number of injected rats in heat	% injected rats in heat
R.U.	mg.			
10	0.5	9	4	44
10	0.8	6	6	100
25	0.5	8	4	50
25	0.8	7	6	86
50	0.5	6	4	67
50	0.8	8	6	75
50	1.0	6	5	83
100	0.5	7	7	100
100	0.8	7	7	100

combined, and 7 of 32 or 22 per cent of the injections of 100 R.U. were followed by heat.

Those spayed-adrenalectomized rats which did not come into heat during the 72 hours following the injection of estradiol benzoate were injected with progesterone with the results seen in Table 4. Whereas 10 R.U. of estradiol benzoate is an effective conditioning dose[2] in the spayed rat, in the spayed-adrenalectomized animal heat can not be induced consistently with less than 100 R.U., although an increase in the amount of injected progesterone tends to increase the percentage

[2] The term "conditioning dose" is used to designate that quantity of estrogen which, when followed after a suitable interval by a sufficient amount of progesterone, will induce heat in all of a group of spayed animals.

response. This is with the obvious exception of the group of 6 animals all of which came into heat after injection of 10 R.U. of estradiol followed by 0.8 mg. of progesterone.

These data strongly suggest a decrease in the sensitivity to estrogen of the genital tissues or neural centers or both in the adrenalectomized animal. Similarly an elevated threshold for the estrus precipitating action of progesterone is demonstrated. The mating behavior induced in spayed-adrenalectomized rats by adequate concen-

TABLE 5. RECORDS OF SPAYED-ADRENALECTOMIZED FEMALE RATS WHICH CAME INTO HEAT FOLLOWING INJECTIONS OF ESTRADIOL BENZOATE ALONE

Animal no.	Results of injections				Survival time	Autopsy findings
	10 R.U.	25 R.U.	50 R.U.	100 R.U.		
13	=		−	∓	Sacrificed at 48 days	Hypertrophied accessory cortical tissue.
22		=	−	±	Sacrificed at 96 days	Regenerated adrenal cortex.
27	−	+		±	Sacrificed at 85 days	Large regen. cortical nodule.
28	−			∓	Sacrificed 30 days	Hypertrophied acc. cortical tiss.
30	+			+	Died 29 days	Large amt. hypert. acc. cort. tiss.
32	+				Sacrificed 28 days	No adrenal tissue found.
35			+	=	Sacrificed 63 days	Large regen. cortical nodule.
37			+	−	Died 47 days	No adrenal tissue found.
41			+		Died 17 days	Regen. cortical nodule.
42			+	+	Died 35 days	Hypert. acc. cortical tissue.

+ injection followed by heat
− injection not followed by heat

trations of estradiol and progesterone in all but a few instances had the same normality with regard to intensity and precision of performance seen in properly stimulated spayed but otherwise normal rats. In a small number of cases responses were weak and difficult to elicit, as if a liminal stimulus were operating.

Of the 10 spayed-adrenalectomized females which came into heat following injections of estrogen alone (Table 5), 8 showed regenerated adrenal cortical tissue or hypertrophied accessory cortical tissue at autopsy. The regenerated tissue was generally grossly visible as a

small nodule at the original adrenal site and was verified in histological preparations. The hypertrophied accessory cortical tissue was seen in sections of peritoneum and mesenteries taken at autopsy from the left pararenal region. Animals 32 and 37 gave no evidence of gross adrenal cortical tissue at autopsy. Histological sections of pararenal tissues showed no accessory cortical tissue.

Excluding those animals in which remaining cortical tissue was demonstrated at autopsy, and those which failed to survive the post operative recovery period, 31 spayed-adrenalectomized female rats received 63 injections of estradiol benzoate in varying amounts. Of these injections 2 were followed by heat, one at 10 R.U. and one at 50 R.U. in 2 different females. The possibility can not be excluded that accessory cortical tissue may have been present in these animals in sites other than that taken at autopsy, since accessory cortex is generally acknowledged to have a fairly wide distribution in the rat. In this connection it should be noted that animal 37 survived for a period 70 per cent longer than the mean survival time.

DISCUSSION

There is ample evidence for the progesterone-like action of corticoadrenal products. Torstveit and Mellish (1941) induced mating responses in spayed guinea pigs with aqueous extracts of adrenal cortex. Van Heuverswyn, Collins, Williams and Gardner (1939) obtained similar results with desoxycorticosterone acetate (DCA). They were also able to produce progestational proliferation in the immature rabbit with DCA, the potency of which was $\frac{1}{6}$ to $\frac{1}{10}$ that of progesterone. Robson (1939) inhibited estrus in normal mice with daily injections of 0.1 to 0.2 mg. of DCA.

Thus it would seem that in the spayed rat with adrenals intact progesterone activity may still be present through the medium of the adrenal cortical hormones. Progesterone itself has been isolated from the adrenal cortex by Beall and Reichstein (1938).

The observed hypertrophy of the adrenals during estrus in the normal rat (Andersen and Kennedy, 1932) and in response to threshold estrogenic stimulation in the spayed rat (Bourne and Zuckerman, 1941) suggests the mechanism by which cortical production of progesterone or progestimimetic substances may be stimulated. Levin (1945) has reported a rapid and progressive decrease in cholesterol levels of serum and adrenal glands following administration of diethyl stilbestrol. A possible cause of the disappearance of cholesterol would be its use in the synthesis of active adrenal steroids. Tepperman, Engel and Long (1943) have reviewed the subject of adrenal cortical hypertrophy. Normal rats, according to these investigators, respond to administration of estrogens by adrenal cortical hypertrophy plus evidences of increased cortical activity: an increased nitrogen excretion and an increase in liver nitrogen, glycogen and weight, results

identical with those obtained by administration of cortical hormones. These reactions, as well as the decrease in cholesterol reported by Levin, fail to occur in hypophysectomized animals, indicating that the effects of estrogens on the adrenal cortex are exerted through the pituitary adrenocorticotrophic factor.

It can thus be postulated that in the spayed female rat injected estrogen indirectly stimulates the adrenal cortex to hypertrophy and release increasing quantities of substances having progesterone activity. If this activity reaches a threshold level, heat results due to the synergistic action of the two hormones.

In the spayed and totally adrenalectomized animal presumably such extra-ovarian progesterone activity is lacking, and injected estrogen of itself is unable to induce heat. This interpretation is based on the assumption that, in the 2 animals which came into heat after injections of estrogen alone, and in which no evidence of persistent cortical tissue was seen at autopsy, widely disseminated accessory cortical tissue may nevertheless have been present and functional. Groat (1943) has described an adrenocortical-like tissue arising by metaplasia in the ovaries of the adrenalectomized ground squirrel, which permits survival of females of this species after removal of their adrenal glands. In the absence of the ovaries the adrenocortical-like cells develop in adjacent areas.

An objection to the tentative interpretation which has been placed on the results of these experiments arises from the fact that, as reported by several investigators, progesterone is capable of sustaining the life of adrenalectomized animals. According to this view the cachexia, asthenia and general debility of animals suffering from adrenal insufficiency would be enough in themselves to diminish the responsiveness of the females to injected estrogens, whereas progesterone through its corticomimetic properties might ameliorate the condition to the extent that the animals would then be capable of responding to estrogenic stimulation.

As reported by various investigators, the quantity of progesterone necessary to prolong the average survival time of adrenalectomized rats ranges from 0.5 mg. per day (Bourne, 1939) to 4 mg. per day (Greene, Wells and Ivy, 1939). In the present experiments the maximum amount of progesterone administered was 1 mg. in a single injection. Whatever ameliorative effects might have been derived from the progesterone were presumably antagonized by the toxic effects of estrogen which have been observed in adrenalectomized rodents by Cavanaugh and Gaunt (1937), Gaunt and Hays (1938) and Pfeiffer and Hooker (1940).

In the present experiments 11 animals died during the 3 days following terminal injections of estradiol. Five animals died during the 3 days following their last progesterone injections, 2 of these becoming moribund about 4 hours after administration of the hormone. During the 5 days following terminal progesterone injections of from 0.5 to

1.0 mg., 13 animals died, of which 6 had been in heat. There was in no case any apparent mitigation of deficiency symptoms by progesterone in the amounts administered. Furthermore, whereas the lowered sensitivity to estrogen was apparent from the initial postoperative injections, the deficiency syndrome did not become obvious until terminal stages.

SUMMARY

Adrenalectomy lowers the sensitivity of spayed female rats to estrogen and to progesterone as demonstrated by tests for sexual receptivity following injections of estradiol benzoate alone and followed by progesterone.

There is some evidence to support a hypothesis that sexual receptivity can be induced in the spayed rat by estrogen alone only in the presence of adrenal cortical tissue as a source of progesterone activity.

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ALTERATIONS IN THE AUTARCEOLOGIC SUSCEPTIBILITY OF THE MOUSE TO EXPERIMENTAL POLIOMYELITIS BY ESTROGENIC SUBSTANCES[1]

G. E. FOLEY AND W. L. AYCOCK

INTRODUCTION

STUDIES of the epidemiologic features of poliomyelitis, such as geographic prevalence (Aycock, 1939), and age distribution (Aycock, 1928), as well as the extensive occurrence of subclinical infection as evidenced by the development of serologic immunity (Aycock & Kramer, 1930), all indicate a widespread and more or less uniform dissemination of the virus, of the order of that of the virus of measles; for example (Aycock, 1934). The extensive occurrence of subclinical infection and the gradation from frank to mild or abortive forms of the clinical disease in themselves indicate that paralysis is selective—the exceptional rather than the usual manifestation of infection with the virus—an event which might be compared epidemiologically with the occurrence of the complication of otitis media in measles (Aycock, 1942a). Certain selectivities in the occurrence of the paralytic disease in a few of the many exposed to the virus indicates that the circumstance determining the outcome of infection resides in the host rather than in parasitic or exposure factors.

The tendency of the paralytic form of the disease to occur in certain families suggests that susceptibility to paralysis may be inherent (Aycock, 1942b), while seasonal and geographic selectivities indicate a physiologic rather than a fixed anatomic character. The association of the paralytic disease with certain constitutional types suggests an endocrine dysfunction (Aycock, 1940) and the suspected greater frequency with which the paralytic disease occurs during pregnancy (Aycock, 1941), in view of the implication of the upper respiratory mucosa as the portal of entry of the virus, suggests that the economy of the estrogenic substances may be concerned with susceptibility.

ESTROGEN IN MONKEYS

Experimentally, castration of immature monkeys (female) (M. rhesus) results in a thinning of the genital (Allen, 1927) and nasal mucosa (Pighini & Porta, 1934). Administration of estrogenic substances to such animals affects recornification (Allen, 1927). Evidence that in monkeys the nasal mucous membranes constitute a barrier

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[1] From the Departments of Preventive Medicine and Epidemiology, Harvard Medical School and School of Public Health, Boston, Massachusetts.

to poliomyelitis virus is supported by the results of treatment of the nasal mucosa with certain chemicals which produce a "blockade" against experimental infection by intranasal instillation of the virus (Flexner & Amoss, 1920, Armstrong, 1936, Sabin, Olitsky & Cox, 1936). It has been further shown that immature castrate (female) monkeys developed poliomyelitis more promptly and more frequently following intranasal instillation of the virus than did similar animals prepared by preliminary administration of estrin (amniotin or diethylstilbestrol) (Aycock, 1937, 1940). That estrogens affect a "barrier" to the virus, or some change in the cellular affinities to the virus is suggested by the observation that monkeys rendered resistant to intranasal virus by estrogen-treatment do not develop subclinical immunity (Aycock, 1936) nor do they exhibit increased resistance to intracerebral injection of the virus (Aycock).

In recent years several viruses of the poliomyelitis group have been passed successfully to smaller laboratory animals. Armstrong (1939) established the now well-known Lansing strain first in cotton rats and then in mice. Armstrong (1941) and later Plotz, Reagan and Hamilton (1942) adapted the Lansing strain to Syrian hamsters. Jungeblut and Sanders (1940) reported the isolation of a strain (SK) which was adapted to cotton rats and white mice from the eleventh monkey passage. Jungeblut, Sanders and Feiner (1942) later reported that this virus was capable of producing paralysis in guinea pigs and albino rats as well as in cotton rats and white mice, either by intracerebral, intraperitoneal, intravenous, intranasal or subcutaneous administration, or by gastric gavage. Wolf (1942) and Ehrich and Foster (1944) reported that the experimental disease produced in mice by these various strains of viruses was similar pathologically to poliomyelitis in man with respect to the nature and distribution of lesions. More recently, Jungeblut and Dalldorf (1943) isolated a strain (MM) of virus from a fatal case (human) of poliomyelitis which was adapted from monkeys to Syrian hamsters, cotton rats and white mice, either by intracerebral or intraperitoneal injection. The availability of strains of poliomyelitis virus which produce infection in animals following peripheral administration suggested the possibility of confirming previous observations on the effect of estrogen treatment on intranasal instillation of the virus in monkeys. Preliminary experiments on mice with the Lansing strain of poliomyelitis virus indicated, as did previous monkey experiments, that the enhanced resistance induced by estrogen treatment could not be demonstrated with direct intracerebral injection of virus.

PRESENT EXPERIMENTS

The virus used in the present experiments was the Mouse-Hamster strain[2] used by Dalldorf and Whitney (1943) in their experiments on

[2] We wish to thank Dr. Gilbert Dalldorf, formerly of Grasslands Hospital, Valhalla, New York for his kindness in supplying this virus.

the interference phenomenon in experimental poliomyelitis. Young Syrian hamsters and white mice are both susceptible to intraperitoneal inoculation with this virus.[3,4] The virus has been successfully adapted to intranasal instillation in white mice in this laboratory by repeated daily instillations of a 5% suspension of infected mouse brain (intraperitoneal mouse) in saline, and to date has been carried through five intranasal passages without loss of ability to produce paralysis when administered intraperitoneally. Intranasal instillation or intraperitoneal injection in white mice is followed in 2–8 days by the development of a typical flaccid paralysis, death ensuing 24–48 hours after appearance of paralytic symptoms. It was exceptional for a paralyzed mouse to survive or recover. The effect of treatment with estrogen on the result of intraperitoneal injection and intranasal instillation of this virus was studied in normal male and female and castrate female mice.

In some experiments castrate and normal white mice 15–20 gm. in weight were treated with 0.01–0.07 mg. of alpha-estradiol dipropionate (Progynon DP)[5] administered subcutaneously as 4–7 daily doses of 0.1 mg. in 0.1 ml. of sesame oil, while in others preparatory treatment consisted of 0.08–0.14 mg. diethylstilbestrol[6] administered subcutaneously as 4–7 daily doses of 0.02 mg. in 0.1 ml. of peanut oil. The response to estrogen therapy was checked by vaginal smear (Papincolaou & Shorr, 1936).

INTRAPERITONEAL VIRUS

Following this preparatory treatment, castrate and normal estrogen-treated and similar numbers of castrate and normal un-treated mice were given a single intraperitoneal injection of Mouse-Hamster virus. The dose of virus varied from 0.05–0.0125 ml. in different experiments, and was prepared from a 10% suspension of intraperitoneally infected mouse-brain in such a manner that the recorded dose was contained in a volume of 0.5 ml. After injection of the virus, the treated mice received 0.01 mg. doses of alpha-estradiol dipropionate or 0.02 mg. diethylstilbestrol (in different experiments) subsutaneously at 48 hour intervals. All animals were observed for a minimum of

[3] Serum known to protect against at least 1,000 M.L.D. of the Lansing strain of poliomyelitis virus does not protect mice against the M-H virus. Formalinized M-H virus failed to protect mice against Theiler's GD V11 virus. On the other hand there were certain immunologic similarities between the M-H and Theiler's GD V11 as well as Jungeblut's SK strains. We wish to thank Dr. W. McD. Hammon, George William Hooper Foundation, University of California, who did the immunologic studies with the M-H virus.

[4] Since this paper was submitted for publication, Dalldorf, G. and Whitney, E. (Proc. Soc. Exper. Biol. & Med., 59: 150. 1945) reported that cross-immunity and neutralization tests indicate a relationship between this virus and the Lansing poliomyelitis virus but not with Theiler's original or low virulence strains. Complement-fixation tests relate this virus to Theiler's GD V11 strain.

[5] We wish to thank Dr. W. R. Bond, Schering Corporation, for his kindness in supplying this product.

[6] E. R. Squibb & Sons product.

21 days. Since the results of various experiments with different doses of virus and either estrogenic substance were essentially the same, all experiments are summarized in Table 1. There was no significant difference in the mortality observed in the estrogen-treated and untreated (castrate or normal) mice following intraperitoneal injection of the virus.

INTRANASAL EXPERIMENTS

The mice used in the intranasal experiments were treated in the same manner as those used for the intraperitoneal experiments. Fol-

TABLE 1. THE EFFECT OF ESTROGENIC SUBSTANCE[1] ON EXPERIMENTAL POLIOMYELITIS IN MICE: INTRAPERITONEAL INJECTION OF M-H VIRUS

No. Mice	Dose of virus	No. deaths	Mortality
Treated Castrates[2]			
12	0.05 ml	12	100.0
12	0.025 ml	12	
Treated Normals[3]			
12	0.05 ml	12	
12	0.025 ml	12	84.0
30	0.0125 ml	21	
Total 78		69	88.0
Untreated Castrates[2]			
12	0.05 ml	11	88.0
12	0.025 ml	10	
Untreated Normals[3]			
12	0.05 ml	9	
12	0.025 ml	11	84.0
30	0.0125 ml	25	
Total 78		66	85.0

[1] Alpha-estradiol dipropionate or diethylstilbestrol
[2] All female
[3] Equal numbers, male and female

lowing preparatory treatment with either alpha-estradiol dipropionate or diethylstilbestrol, castrate female and normal male and female estrogen-treated and similar numbers of castrate female and normal male and female un-treated mice were given intranasal instillations of 0.1 ml. of a 10% or 2.5% suspension of intranasally infected mouse-brain. Instillation was repeated daily until the first animal in a given experiment developed paralysis. All mice were observed for a minimum of 21 days. Since the results of individual experiments were essentially the same, all intranasal experiments are summarized in Table 2. The mortality in the estrogen-treated (castrate or normal) mice was significantly less than that observed in un-treated (castrate or normal) mice following intranasal instillation of the virus. In these experiments, the differences between the mortality of test animals and controls were consistent for the various doses of virus.

DISCUSSION

Intracerebral injection of the virus in the experimental disease may be an unsatisfactory method with which to assay variations in host reaction to infection since the locus of resistance may lie outside the central nervous system. In monkeys for instance, intracerebral injection of poliomyelitis virus is almost universally followed by the development of the frank disease. That direct introduction of the virus into the central nervous system may mask host differences which

TABLE 2. THE EFFECT OF ESTROGENIC SUBSTANCE[1] ON EXPERIMENTAL POLIOMYELITIS IN MICE: INTRANASAL INSTILLATION OF M-H VIRUS

No. mice	Dose of virus	No. deaths	Mortality
Treated Castrates[2]			
15	0.01 ml—4 days	7	37.0
12	0.01 ml—5 days	2	
Treated Normals[3]			
12	0.01 ml—3 days	4	29.0
12	0.025 ml—8 days	3	
Total 51		17	33.0
Untreated Castrates[2]			
22	0.01 ml—4 days	14	63.0
18	0.01 ml—5 days	11	
Untreated Normals[3]			
12	0.01 ml—3 days	6	46.0
12	0.025 ml—8 days	5	
Total 64		36	56.0

[1] Alpha-estradiol dipropionate or diethylstilbestrol
[2] All females
[3] Equal numbers, males and females

are apparent when other routes of injection are employed is well known. For example, the marked effect of age on the susceptibility of mice to the viruses of equine encephalomyelitis (Olitsky, Cox and Syverton, 1934), vesicular stomatitis (Sabin and Olitsky, 1937), and rabies (Casals, 1940), are demonstrable by peripheral but not by intracerebral injection. In previous experiments, similar to those recorded here estrogen-treatment did not affect the resistance of mice to the Lansing strain of poliomyelitis virus, probably because intracerebral injection of the virus was employed.

The failure of estrogen-treatment to enhance resistance against intraperitoneal infection with poliomyelitis virus, as it does against hemolytic streptococcal infection in mice (Foley and Aycock, 1944) suggests different modes of dissemination for the two infectious agents within the peritoneum, and in a sense, illustrates a selectivity of host variation, in that a single substance exhibits different effects on the same host with respect to two widely different infectious agents.

The failure of estrogen-treatment to protect mice against intra-

cerebral and intraperitoneal injection of virus, while protection was afforded against intranasal instillation is similar to the results obtained in monkeys (Aycock, 1936). The enhanced resistance in both estrogen-treated monkeys and mice to intranasal instillation of the virus suggests that estrogen-treatment operates through a "barrier effect" to the virus (upper respiratory mucous membranes) or through some change in cellular affinity to the virus; such a change perhaps residing outside the central nervous system. That autarceologic variation in the upper respiratory mucous membranes does constitute a factor in the resistance or susceptibility to poliomyelitis is well established by the occurrence of the bulbar type of the disease following surgical procedures involving the upper respiratory tract (Aycock, 1942c). The suspected increased frequency of poliomyelitis in pregnancy (Aycock, 1941) and the relationship of a subclinical endocrinopathy (Aycock, 1940) to the paralytic form of the disease suggests that in man, autarceologic susceptibility may be dependent upon some inherent endocrine fault.

CONCLUSION

Treatment of castrate female and normal male and female mice with alpha-estradiol dipropionate or diethylstilbestrol enhanced resistance against infection by intranasal instillations of a suspension of Mouse-Hamster poliomyelitis virus. Similar treatment did not enhance resistance against infection with the same virus when the virus was injected intraperitoneally. Other experiments with the Lansing strain of poliomyelitis virus showed that the enhancing effect of estrogen on resistance to infection could not be demonstrated by intracerebral injection of the virus in mice. These results are similar to those obtained earlier in monkeys—estrogen-treatment enhanced resistance against infection by intranasal but not by intracerebral administration of virus.

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THE EFFECTS OF THE SYNTHETIC AND NATURAL HORMONE OF THE ADRENAL CORTEX ON MELANIN DEPOSITION IN ADRENALECTOMIZED BLACK RATS FED DIETS ADEQUATE AND DEFICIENT IN THE FILTRATE FACTORS OF VITAMIN B[1]

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IN A PREVIOUS STUDY (Ralli and Graef, 1943) we reported the stimulating effect of adrenalectomy on hair growth and melanin deposition in black rats that were maintained on diets either deficient in the filtrate factors of vitamin B, or diets supplemented with a rice bran extract. The effect of adrenalectomy was particularly striking in the rats receiving the deficient diet because of the fact that filtrate factor deficiency is associated with graying of the fur which is due to a depression of melanin deposition and an associated atrophy of the hair follicles and bulbs. Following adrenalectomy the effects of the deficient diet were reversed.

In the present study, the role of the adrenal cortex in the reaction was further investigated by administering to the rats, following adrenalectomy, either desoxycorticosterone acetate or the naturally occurring adrenal cortical hormone. As in the previous study, 30 to 35 day old black rats were placed on the filtrate factor deficient diet (Ralli and Graef, 1943). The diet was continued for 28 to 32 days by which time definite evidences of achromotrichia were present. The rats were then adrenalectomized and after the operation some of the rats were continued on the deficient diet and others were given the diet supplemented with a rice bran concentrate or with calcium pantothenate. Skin samples were taken at suitable intervals for microscopic study. A 1% solution of NaCl was given *ad lib* post-operatively to all but 2 groups of rats.

One hundred and thirty-five rats were adrenalectomized. The arrangement of the experiments is given in Table 1. Groups 3 and 7 did not receive the 1% NaCl solution. Groups 1, 2 and 3, totalling 84 rats, were maintained on the deficient diet both before and after operation. Following adrenalectomy the rats in Group 1 were injected daily with 0.4 cc. of an adrenal cortical extract,[3] and the rats in Groups 2 and 3

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received daily injections of 0.5 mgms. of desoxycorticosterone acetate (DOCA).[4] The diet of the rats in Groups 4 and 5 was supplemented with a rice bran concentrate[5] following operation. The 13 rats in Group 4 were injected daily with the adrenal cortical hormone, and the rats in Group 5 were injected daily with DOCA. The diet of the rats in Groups 6 and 7 was supplemented with calcium pantothenate[6] (4 mgms. daily), and in both groups DOCA was injected daily. The rats in Group 7 did not receive any NaCl.

A group of 69 rats serving as non-operative controls were treated with the same diet and hormone therapy as were the adrenalectomized animals. The effect of varying the NaCl concentration in the drinking water was studied in some of the rats.

RESULTS

In adrenalectomized rats maintained on filtrate factor deficient diets a bluing of the skin becomes evident on the 5th to 7th day (Ralli and Graef, 1943). When the adrenal cortical hormone was injected daily following adrenalectomy, the onset of the coloration was postponed slightly, occurring about the 8th to 10th day. However, once the coloration had begun, it increased and deepened reaching a peak on the 15th to 18th day. Grossly our impression was that the skin did not appear as blue as in animals not receiving the hormone. There was much less regrowth of fur over the areas shaved at the time of operation than had occurred in the non-hormone treated rats. In the rats treated with DOCA (Groups 3 and 4), the prevention of the stimulating effect of adrenalectomy was more striking. Gross evidence of melanin deposition as reflected in the uniform blue color of the skin was not observed. On or about the 9th to the 10th day, traces of color were

ARRANGEMENT OF EXPERIMENTAL GROUPS OF ADRENALECTOMIZED RATS

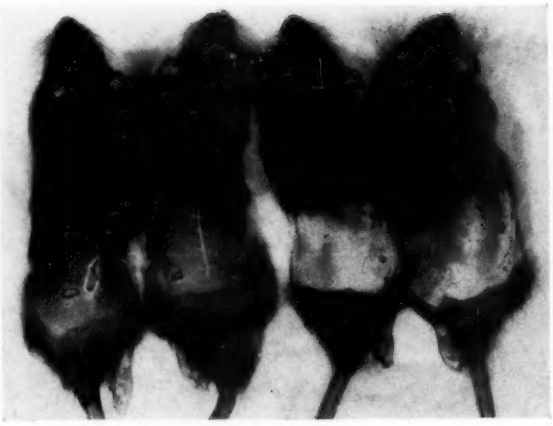
Group	Diet	Dietary post-op. supplement	Hormone	No. of rats
1	FFD	None	Cortin	24
2	FFD	None	Doca	43
3	FFD	No NaCl	Doca	17
4	FFD	Rice Bran Extract	Cortin	13
5	FFD	Rice Bran Extract	Doca	13
6	FFD	Calcium Pantothenate	Doca	4
7	FFD	Calcium Pantothenate No NaCl	Doca	21

[4] Supplied by the Hoffman LaRoche Co., Nutley, N. J.
[5] Supplied by the Galen Co., Berkeley, California.
[6] Supplied by the Walker Vitamin Co., Mt. Vernon, N. Y.

visible in spots in some of the rats but this was never uniform or deep. Regrowth of fur over the area shaved at operation did not take place. The absence of NaCl in the drinking water had no visible effect on melanin deposition.

When the diet was supplemented with a rice bran concentrate and the rats received the adrenal cortical hormone (Group 4), there was no visible retardation in the onset of bluing of the skin and the intensity of the color was as great as in untreated rats. There was, however, retardation of hair growth which was in sharp contrast to animals not receiving cortin. The injection of DOCA (Group 5) again depressed melanin deposition in spite of the dietary supplement and in none of the rats was any consistent or deep bluing of the skin apparent. Regrowth of fur was also arrested. Groups 6 and 7 received calcium pantothenate, lack of which is considered to be the specific cause of achromotrichia (Gyorgy and Poling, 1940; Unna, Richards and Sampson, 1941). The daily injection of DOCA was again effective in preventing the appearance of consistent or deep bluing of the skin that follows adrenalectomy in non-hormone treated rats and that is especially marked when the previously deficient diet is supplemented with calcium pantothenate. The administration of 1% NaCl did not have any demonstrable effect on the gross appearance of the skin. The growth of fur was also inhibited in the rats in these groups. Figure 1 illustrates the differences in the color of the skins in adrenalectomized rats receiving hormone therapy as compared to rats not treated with the hormones. Of the 4 rats shown (A 26-14, A 26-33, A 27-31 and A 27-21), A 26-14 had been receiving the filtrate factor deficient diet before adrenalectomy and was continued on the diet after adrenalectomy but *did not* receive any hormone. The photograph was taken 15 days after adrenalectomy and in this animal there is the usual blue appearance of the skin. The next rat (A 26-33) was a litter mate kept on the same diet, but following adrenalectomy the animal received injections of the adrenal cortical hormone daily. The photograph, also taken 15 days after adrenalectomy, shows the bluish color of the skin, slightly less intense but still uniform. Rat A 27-31 received the deficient diet and daily injections of DOCA following adrenalectomy. The photograph, 12 days after adrenalectomy, demonstrates the inhibitive effect of the synthetic hormone on melanin deposition as reflected in the skin. The 4th rat (A 27-21) received the diet *supplemented* with the filtrate factors after adrenalectomy and also received injections of DOCA. Although there is some coloration of the skin, it is spotty and not the intense blue that has appeared when adrenalectomized rats received the filtrate factors.

The effects of hormone therapy on the unoperated control rats are easier to appreciate if the influence of the deficient and supplemented diets on hair growth and pigmentation are briefly described. When young black rats have been maintained on a filtrate factor deficient diet for 30 days, graying of the fur occurs. The pattern of the graying



A26-14	A62-33	A27-31	A27-21
F.F.D.	F.F.D.	F.F.D.	Diet c Filtrate Factors
No Hormone	Cortin	D.O.C.A.	D.O.C.A.

is not identical in all animals, as has been pointed out, (Ralli and Graef, 1943) sometimes being confined to the back, sometimes to the hides and the coat. If after 30 days on the deficient diet the animal is shaved, the skin will usually be pink with no pigment bands. Fur will begin to regrow over the shaved area on the 10th to 12th day. The regrowth is slow and the fur will always regrow gray (Mushett and Unna, 1941). If, at the time of shaving, the deficient diet is supplemented with a rice bran extract or calcium pantothenate, regrowth of fur will begin about the 10th to 12th day, the fur that grows in will be fairly well pigmented and its texture will be better than in the rat continued on the deficient diet. A second shaving in the rats continued on the deficient diet results in no regrowth of fur due to the progression of the atrophy of the hair apparatus. 1% NaCl in the drinking water of rats receiving the deficient diet had no striking effect on the pigmentation of the regrown fur, but in the rats receiving calcium pantothenate and 1% NaCl, the fur was definitely better pigmented. The effect of NaCl was tested further by increasing the concentration in the drinking water to 2%. This resulted in some dehydration of the rat and had a very distinct effect on the pigment bands in rats receiving calcium pantothenate. The visible pigmentation of the normal pigment bands was intensified and there was a diffuse bluing of the skin. This color was not as evenly distributed as in the adrenalectomized rats but it was quite definite.

The injection of the naturally occurring adrenal cortical hormone had no visible effect on either the color of the fur or the time required for the regrowth of fur in control rats receiving either the deficient or the supplemented diets. However, when DOCA was injected daily, the regrowth of fur was retarded. The effect of DOCA was more distinct in the control rats receiving the supplemented diet as in these rats the regrown fur is pigmented. The injection of DOCA retarded the onset of visible regrowth of fur until about the 15th day and in many animals the regrown fur was scanty. The fur that did regrow was not uniformly pigmented. In general, the depressive effect of DOCA on the hair apparatus was not as striking or as uniform in the unoperated rats as in the adrenalectomized animals.

HISTOLOGIC OBSERVATIONS OF THE SKIN OF THE ADRENALECTOMIZED RATS

In the rats treated with adrenal cortical extract or desoxycorticosterone acetate after adrenalectomy, the hyperplastic response of the hair bulbs and the associated deposition of melanin, usually present after adrenalectomy, was diminished. Whereas proliferation of the hair buds had been discernible as early as 72 hours after adrenalectomy in both normal and deficient rats, in the animals receiving either form of hormone therapy the response was often postponed until 5 to 9 days after operation. The changes observed were much more striking

in the DOCA treated animals. The proliferation that did take place was uneven and definitely less marked than in the rats that were not given hormone therapy. Furthermore, melanin deposition in the hormone-treated animals was only present to a moderate degree and in the rats receiving DOCA was quite often completely absent in some of the hair bulbs. Melanin deposition as previously reported was uniform in animals that were not given hormone therapy and the deposition continued until it reached a peak at about 14 to 17 days; after this period fading took place. Although the proliferative response was just as inconstant as the melanin deposit in hormone treated animals, it continued for about the same period as in untreated adrenalectomized animals. Some skin samples taken 22 days after operation showed small "resting" bulbs and hair follicles with a little pigment present in some. The hair shafts, likewise, were unevenly pigmented and this was especially notable in the skin of animals that had developed marked achromotrichia as a result of the deficient diet. In rats not given hormone therapy, skin samples 22 days after operation usually disclosed many more hyperplastic bulbs still rich in melanin. When the diet was supplemented with the filtrate factors after adrenalectomy, the injection of DOCA in the rats also inhibited the usual proliferative response to adrenalectomy. Some response, however, was visible as early as 72 hours after operation but pigment deposition did not bear any relation to the degree of hyperplasia of the hair buds and follicles. Some very large cellular bulbs contained no pigment in their bases or hairs, a finding that was not encountered in the adrenalectomized animals not treated with hormone.

MICROSCOPIC APPEARANCE OF THE HAIR APPARATUS IN THE CONTROL RATS (NOT ADRENALECTOMIZED)

Since a deficiency of the filtrate factors causes a depression of melanin deposition and an associated atrophy of the hair follicles and bulbs in normal rats, it is obvious that the administration of the adrenal cortical hormone to rats on such diets would have very little demonstrable effect on melanin deposition. The microscopic appearance of the skins of the "deficient" unoperated rats receiving either cortin or DOCA did not show any greater atrophy of the hair apparatus or any further decrease in melanin deposition than was already present as a result of the dietary deficiency. An occasional hair follicle was visible and the hair bulbs were scattered sparsely throughout the subcutaneous layers. The hair apparatus was atrophic and melanin deposition was irregular.

When the diet was supplemented with calcium pantothenate, small, young hair follicles and bulbs were fairly numerous in the area under the cutis and in the deeper areas. There were large proliferating bulbs which were well pigmented as were the hair follicles. Shafts of hairs could be seen in the sections. In the skin sections of rats receiving

the supplement of calcium pantothenate but who also were given DOCA, there was a distinct absence of proliferation of the hair follicles and bulbs. Numerous hair follicles were seen but they were in an arrested state of development and were poorly pigmented. Whereas the skin of the rats not receiving DOCA showed long well pigmented hair follicles with proliferating hair bulbs, the skin sections of the DOCA rats showed definite arrest of the growth of the hair follicles and a decrease in pigmentation.

DISCUSSION

The results of these experiments and those previously reported (Ralli and Graef, 1943) indicate that melanin deposition in the hair

ANALYSES OF THE SKINS OF HORMONE TREATED AND UNTREATED ADRENALECTOMIZED RATS ON FILTRATE FACTOR DEFICIENT DIETS

	F.F.D. Adr.	F.F.D. Adr. + Doca	F.F.D. Adr. + Cortin
No. of rats	10	6	6
Weight—gms.	94	101	96
Survival of exp. condition days	8.4	13.2	9.2
% H^2O skin	67.8	71.8	69.4
Phenol Mg/Gm tissue	0.498	0.168	0.580
Reducing power Meq. $KMnO_4$/Gm	0.033	0.014	0.033
Lipid			
Gm/Gm T.	0.009	0.021	0.007
Iodine #	41.2	39.8	38.3
Tyrosinase MgM/Gm tissue	28.6	27.7	23.5
Melanin MgM/Gm tissue	23.5	11.9	19.7

From Spoor and Ralli, Endocrinology, 1944.

bulbs and follicles of black rats and the associated hyperplasia of the hair apparatus is influenced by the adrenal cortex and its hormones. Removal of the adrenal gland stimulates hyperplasia of the hair apparatus and increases melanin deposition in the hair follicles and bulbs. This stimulus is so great that it will reverse the atrophy of the hair apparatus and the diminished melanin deposition caused by a diet deficient in the filtrate factors of vitamin B. Desoxycorticosterone acetate will inhibit the effect of adrenalectomy on the hair apparatus and melanin deposition. The naturally occurring adrenal cortical hormone has a much less striking effect and the difference is probably due to the larger amount of the pure steroid made available when DOCA is injected as compared to the concentration in the naturally occurring hormone. Butcher (1939, 1941) also observed that increased hair growth followed adrenalectomy in white rats.

The fact that the feeding of a rice bran concentrate or of calcium pantothenate caused some deposition of melanin in the hair bulbs in spite of hormone therapy suggests that exogenous factors can also

influence melanin deposition. Spoor and Ralli (1944) reported the melanin content of the skins of adrenalectomized rats receiving filtrate factor deficient diets and treated with either DOCA or the adrenal cortical hormone. Their results support the microscopic and gross observations reported in this study and the data from Table 2, quoted from their paper, shows that the melanin content of the skins of rats treated with the natural adrenal hormone or with the synthetic hormone was significantly lower than the melanin content of the skins of non-hormone treated adrenalectomized rats. Desoxycorticosterone acetate had the more striking effect on melanin deposition.

The effect of desoxycorticosterone acetate on melanin deposition was also evident in non-adrenalectomized rats receivnig a diet supplemented with calcium pantothenate. Normally, this supplement will tend to increase melanin deposition and will stimulate hair growth in the pigmented bands of the skin. The administration of DOCA arrested the development of the hair follicles and decreased the amount of melanin. The effect of DOCA was not as marked in the unoperated rats as it was in the adrenalectomized rats because the marked stimulus to hyperplasia of the hair follicles and melanin deposition induced by adrenalectomy affords a better contrast for the inhibiting effect of desoxycorticosterone acetate.

Melanin deposition is also undoubtedly influenced by factors other than adrenalectomy. Among such factors are probably the concentration of sodium chloride in the body fluids and dietary supplements such as calcium pantothenate. Microsopcic section of the skins of the non-operated rats receiving 2% NaCl and the filtrate factor deficient diet and the diet supplemented with calcium pantothenate, were not done. However, grossly the rats receiving both the calcium pantothenate and 2% NaCl showed an increase in the intensity of the normal pigment bands of the skin and a diffuse bluing of the skin, suggesting an increase in melanin deposition. The factors affecting melanin deposition in non-adrenalectomized rats deserves further study.

SUMMARY

The stimulating effect of adrenalectomy on melanin deposition and hair growth in black rats was suppressed when the rats were injected with the adrenal cortical hormone. The synthetic hormone, desoxycorticosterone acetate, was more effective than was the adrenal cortical extract. As previously reported, the stimulus of adrenalectomy was so great that it reversed the atrophy of the hair apparatus caused by feeding the rats a diet deficient in the filtrate factors of vitamin B. In non-adrenalectomized rats fed the deficient diet, the administration of DOCA did not have any discernible effect on the already atrophic hair follicles or on the decreased melanin content of the cells. However, the effects of DOCA were evident in normal rats receiving a rice bran extract or calcium pantothenate. These supple-

ments in the diet will maintain the hair apparatus in its normal state with a normal deposition of melanin. When DOCA was injected daily in such rats, the normal growth of the hair follicles was definitely suppressed and the melanin content of the cells was decreased. Analyses of the melanin content of the skins of such rats showed a decrease in their melanin content. The observations furnish experimental proof of the effect of the adrenal cortex and its hormones on melanin metabolism, confirming the earlier report of the stimulating effect of adrenalectomy on melanin deposition and hyperplasia of the hair apparatus, and showing that this effect can be significantly depressed by the daily administration of the synthetic adrenal cortical hormone, desoxycorticosterone acetate.

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METABOLISM OF THE STEROID HORMONES: THE ISOLATION OF AN ANDROSTENOL-3(α)-17-ONE, AN ANDROGEN, FROM THE URINE OF A GIRL SUFFERING FROM VIRILISM[1]

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WOLFE, FIESER AND FRIEDGOOD (1941) isolated an androstenol-3(α)-17-one from the urine of a ten-year-old girl who was suffering from a malignant adrenocortical tumor. The evidence presented by these investigarors did not establish the position of the double bond, but indicated that it was in one of four positions: 6,7-, 7,8-, 9,11-, or 11,12-. They isolated this substance in a concentration of 8 mg. per liter. We have been able to isolate the same androstenol-3(α)-17-one from the urine of a woman suffering from virilism, and to show that this substance is an androgen.

The patient (Wisconsin General Hospital, No. 127483) was first seen at age 17½ years because of primary amenorrhea and a suspicion of masculine traits. An older sister had a very similar condition and had been studied in the same hospital a few years earlier. The important features on physical examination were a rather large and square type of jaw, heavy musculature about the neck and shoulders, lack of mammary development; there was slight hirsutism on the upper lip and chin, profuse hair growth in the axillae, and the hair pattern on the lower abdomen and about the pubis was of a masculine type. There was an increased amount of hair on the legs. The voice was deep, husky and rather typical of a young adult male. Blood pressure was 130/80. Examination of the genitalia revealed a markedly enlarged clitoris, about 3 cm. in length, with a definite prepuce. A small vaginal opening was demonstrated and lipiodol was introduced into the cervix which made it possible to demonstrate a uterine cavity filled with opaque medium, which did not extend into tubes on either side. The uterine cavity was very small. X-ray of the skull was not

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remarkable. X-ray of the pelvis suggested a female type of bony structure. At laparotomy under general anesthesia a small uterus was found, the tubes and ovaries appeared of approximately normal size but with very thick ovarian capsules and the ovaries were polycystic in type. The adrenals seemed normal to palpation. Biopsies were obtained from the ovaries and on examination showed dense stroma with a number of small follicles. The clitoris was amputated.

Repeated smears of the vaginal mucosa failed to show any evidence of estrogenic activity. Assays of the gonadotrophic hormone in 12-hour urine specimens showed this material present but not in greater amounts than are conventionally found in urine of healthy women in the late second or the third and fourth decades. Estimation of the total neutral 17-ketosteroid excretion varied between 63 and 144 mgs. per 24 hours with characteristic values in the range between 80 and 90 mgs.

Attempts to stimulate the ovaries by large doses of known active pituitary gonadotrophin were without demonstrable results as far as estrogenic activity in the vaginal mucosa is concerned. Very large doses of estrogen were necessary to obtain minimal response in the same way. So, for example, 50,000 international units of estrogenic substance in oil intramuscularly every three days gave very slight response but made the breasts begin to grow.

A total of 25.4 liters of urine was collected with toluene as the preservative and hydrolyzed with 10% hydrochloric acid by volume for 10 minutes under the reflux. The hydrolyzed urine was cooled immediately under the tap and extracted in a separatory funnel three times with fresh portions of benzene (1 part benzene to 4 parts urine). The combined benzene layers were washed with small portions of saturated sodium bicarbonate and then with water. The benzene was evaporated in vacuo and the oily residue dissolved in ether and extracted with 10% sodium hydroxide. The neutral fraction contained 105 mg. of 17-ketosteroids per liter. This fraction was separated with the Girard-Sandulesco reagent T into the ketonic and nonketonic fractions.

The ketonic neutral fraction ($\frac{3}{4}$ of total) was acetylated with acetic anhydride and pyridine, dissolved in 60 cc. of a mixture of benzene (1 part) and petroleum ether (3 parts) and adsorbed on a column of 30 gm. Al_2O_3 (Brockmann). The first eluate contained no residue. A further quantity of 60 cc. of benzene (1 part) and petroleum ether (3 parts) also yielded no residue. When 100 cc. of a mixture of equal parts of benzene and petroleum ether was used, a batch of colorless crystals was obtained. One crystallization from 95% ethanol followed by recrystallization from absolute methanol yielded 105 mg. of a substance, m.p. 179–180° C.[2] A sample dried at 100° C. for 1 hour at 1 micron was analyzed.

[2] All melting points are uncorrected and were determined with the Fischer-Johns apparatus.

	C	*H*
Calculated for $C_{21}H_{30}O_3$	76.31	9.15
Found	75.79	9.17

When this substance was mixed with a sample of androsterone (m.p. 183° C.) the melting point was depressed (m.p. 140–150° C.).

Twenty milligrams of the compound were treated with 1.8 cc. of 95% ethanol and 0.3 cc. of 10% NaOH and heated in a water bath for ½ hour. A compound was isolated which melted at 181–181.5° C. When it was mixed with the substance obtained originally from the column, the melting point was depressed to 143–150° C. Presumably the original compound was an acetate. A mixture of the free compound, m.p. 181–181.5° C., with a sample of androsterone, m.p. 183° C., melted at 170–175° C. When the free compound was mixed with a sample of the androstenol-3(α)-17-one (m.p. 178–179° C.), isolated by Wolfe, Fieser, and Friedgood (1941), no depression in melting point was obtained (m.p. 179–180° C.).[3]

ASSAY FOR ADRENAL CORTICAL ACTIVITY

An assay for adrenal cortical activity was performed using adrenalectomized rats in a cold test previously described (Dorfman *et al.*, 1944). No activity was found at a dosage of 3.3 mg. of the acetate per rat. By this method significant responses are usually obtained with 64 micrograms of 11-dehydrocorticosterone.

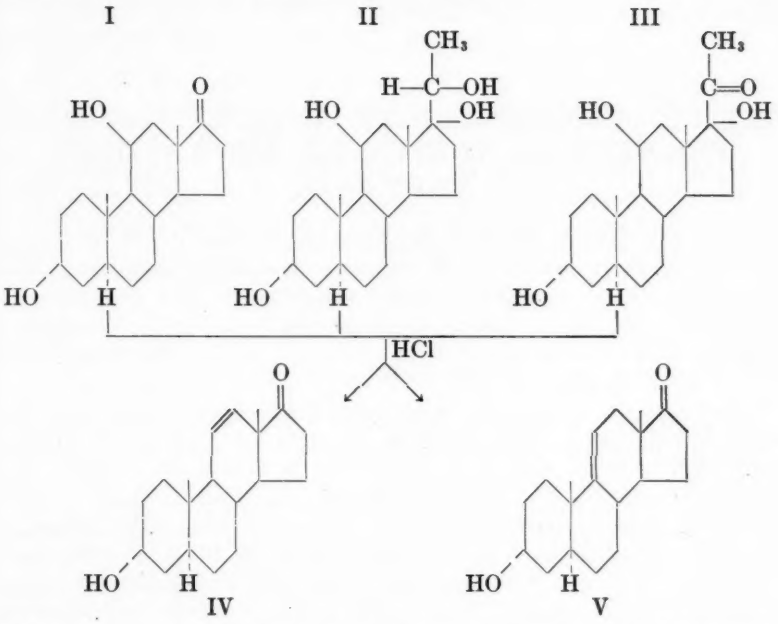
ASSAY FOR ANDROGENIC ACTIVITY

Both the androstenol-3(α)-17-one and its acetate were assayed for androgenic activity using an inunction technic on the chick's comb (Frank *et al.*, 1942). Using twenty chicks for each of the assays, the acetate showed an activity of 2.7 International Units per mg. while the free compound assayed 2.9 I.U. per mg.

DISCUSSION AND CONCLUSION

A yield of 91.3 mg. of androstenol-3(α)-17-one (105 mg. of acetate) was obtained, corresponding to 4.8 mg. of the free compound per liter. This figure is of course a minimum one. Wolfe, Fieser, and Friedgood suggested that androstenol-3(α)-17-one omay be the C_3-epimer of an androstenol-3(β)-17-one, prepared by dehydration of a substance derived from one of the cortical steroids having an hydroxy group at C-11. If this androstenol-3(α)-17-one is a metabolite of an adrenal steroid having a C-11 hydroxy group, the nuclear double bond may be either at 9,11, at 11,12, or a mixture of both. Thus the urinary metabolite may have been a C-11-hydroxy compound such as Compound I, II, or III.

[3] We are grateful to Prof. Fieser for the sample of androstenol-3(α)-17 one.



When subjected to hydrolysis in the presence of hydrochloric acid, Compounds I, II, or III may give rise to either $\Delta^{9:11}$-androstenol-3(α)-17-one (V), $\Delta^{11:12}$-androstenol-3(α)-17-one (IV), or a mixture of both.

SUMMARY

An androstenol-3(α)-17-one was isolated (4.8 mg. per liter) from the urine of a girl suffering form virilism. This substance appears to be identical with that isolated by Wolfe, Fieser, and Friedgood from the urine of a ten-year-old girl suffering from a malignant adrenocortical tumor. The androstenol-3(α)-17-one is androgenic and assayed 2.9 I.U. per mg. Its acetate was also androgenically active and assayed 2.7 I.U. per mg. No adrenal cortical activity could be demonstrated by means of a cold protection test.

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EFFECT OF TESTOSTERONE PROPIONATE ON SEMINAL VESICLES OF JUVENILE AND CASTRATE MALE RATS

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INTRODUCTION

SINCE 1929, the capon or chick comb method has been used for the assay of androgens, (Gallagher & Koch, 1929, 1935; Dorfman & Greulich, 1937; Frank, Klempner, Hollander & Kriss, 1941, 1942). Recently, however, it has been reported that this method does not yield accurate quantitative results, (Duff & Darby 1941; McCullagh & Guillet, 1941; Hoskins, Beach, Coffman & Koch 1941). From a practical point of view it is not very desirable, for it is difficult and expensive to maintain a large flock of birds under conditions necessary for assay work, (Starkey, Grauer and Saier, 1940). The accessory sex organs of the mammal have also been used as a criterion of androgenic activity, (Callow & Deanesly, 1935, Korenchevsky, 1932, Bülbring & Burn, 1935). Greene & Burrill, 1940, 1941 have described a method for the assay of androgens by using immature male rats, a technique which has been in use in our laboratory for the past two years.

While the juvenile rat method has been satisfactory for most investigational problems it had several disadvantages as a routine test for control work. When the seminal vesicles were removed forty-eight hours after the injection of the hormone it was impossible to distinguish accurately between different concentrations unless large numbers of rats were used. Furthermore, it has necessitated our having to breed a large number of rats in order to have on hand enough immature rats between the ages of 20 to 22 days to carry out the assay. This was not always possible and resulted in considerable delay.

The data we are presenting is divided into two parts: (1) the extension of time from forty-eight to seventy-two hours in the juvenile method in an attempt to attain the maximum growth of the seminal vesicles; (2) the development of a method which would be reliable and accurate and at the same time practical for commercial laboratories. This has involved the use of castrate male rats and we have determined: (a) the age at which rats can be castrated without having to wait for seminal vesicle regression; (b) a dose response curve and

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(c) the optimum time for removal of seminal vesicles following the injection of various doses of hormone.

METHODS

Immature Rats (Wistar Strain)—The method outlined by Greene and Burrill has been followed as closely as possible. All rats were bred and raised in our own laboratory under standard conditions and only those rats weighing 25 to 32 grams between the ages of 20 to 22 days were used. The doses of testosterone propionate ranged from 0.1 mgm. to 1.0 mgm., per animal, and were given in a volume of 0.1 cc. sesame oil. All injections were given subcutaneously with a sterile syringe and needle.

Castrate Rats (Wistar Strain)—Except in one experiment, (Table 3) all rats were castrated at 27 to 31 days of age and were then allowed to recover fully from the operation before being used. Doses of testosterone propionate ranged from 0.1 mgm. to 1.0 mgm. per rat and the interval of injections was from 7 to 350 days after castration.

Removal of Seminal Vesicles—The seminal vesicles were removed at either forty-eight, seventy-two, ninety-six or one-hundred and twenty hours after the injection of testosterone propionate. Under the dissecting microscope the coagulating glands were teased from the seminal vesicles. The latter were then incised near the point of entrance into the ejaculatory duct, immersed in saline, placed on a blotting paper for a few seconds and then weighed on a Roller-Smith milligram balance.

RESULTS

Forty-Eight and Seventy-Two Hour Response to Testosterone Propionate in the Immature Rat

Greene and Burrill (1941) demonstrated that in the immature rat there was a dose response relationship for doses of testosterone propionate ranging from 0.005 mgm. to 0.250 mgm. with significant differences between alternate but not between adjacent groups. Using a somewhat higher dose range (0.1 to 1.0 mgm.) we also found a dose response relationship but with significant differences between adjacent as well as alternate groups (Table 1). This discrepancy between adjacent groups might be due to the fcat that in our experiments a larger number of animals were used and the dose increment was somewhat higher. Even though there was a significant difference between adjacent groups, the weight increment was very small between 0.1 and 0.25 mgm. For this reason a longer time interval was selected in the hope that by allowing these doses to exert their influence over a longer period of time, wider differences in seminal vesicle weights could be obtained. Three groups of rats were injected with 0.10, 0.25 and 1.0 mgm. respectively and the seminal vesicle weights determined at the end of seventy-two hours. It will be seen in Table 2 that a considerable increase in seminal vesicle weight occurs when 0.25 and 1.0 mgm. are allowed to act for an additional 24 hours. In the case of 0.25 mgm. the seminal vesicles increased from 13.4 mgm. in forty-eight hours to 19.5 mgm. at seventy-two hours, while with 1.0 mgm. the seminal vesicles

increased from 14.6 to 22.9 mgm. The seventy-two hour response to 0.10 mgm. was slightly less than the forty-eight hour response, i.e., 12.3 to 11.3 mgm. This would indicate that the smaller dose has produced its maximum effect in forty-eight hours.

It can also be observed that at the end of seventy-two hours there were larger differences in the responses to the three doses than at forty-eight hours. The difference between the mean seminal vesicle weights

TABLE 1. FORTY-EIGHT AND SEVENTY-TWO HOUR RESPONSE TO TESTOSTERONE PROPIONATE IN THE IMMATURE MALE RAT

No. Animals	Dose T.P. (mgm.)	Mean S.V. wt. (mgm.)	S.E.	t	Sig. +	Control S.V. wt. (mgm)
48 hour response						
40	0.10	12.3	0.23	2.79	+	4.7
32	0.25	13.4	0.32	2.57	+	4.5
10	1.00	14.6	0.34			4.7
72 hour response						
18	0.10	11.8	0.36	10.55	+	5.0
28	0.25	19.5	0.64	3.82	+	5.1
12	1.00	22.9	0.50			4.8

+ Probability of 0.05 or less considered significant.

TABLE 2. SEMINAL VESICLE WEIGHTS OF NORMAL RATS

Age	22–24 days	27–31 days	32–36 days	40–41 days	50 days
Number of rats	191	16	16	13	12
Average S. V. Weight (mgm.)	4.7	6.3	12.7	19.1	44.3
Average Body Weight (gms.)	28	43	79	95	115

produced by 0.10 and 0.25 mgm. was only 1.1 mgm. at forty-eight hours, but was 7.7 mgm. at seventy-two hours. Between 0.25 mgm. and 1.00 mgm. the difference was 1.2 mgm. at forty-eight hours and 3.4 at seventy-two hours. Thus, in spite of the fact that fewer rats were used in the seventy-two hour method and that the standard error was greater, the "t" tests between adjacent groups showed greater significant differences in case of the seventy-two hour assay method. We did not attempt to obtain a complete dose response curve, but on the basis of the responses to the doses which we used, we may conclude that a seventy-two hour method using juvenile rats would be more satisfactory than the forty-eight hour method, especially if doses of 0.25 mgm. or more are to be used in the assay.

Seminal Vesicle Weights of Normal Rats

It has been reported that there is relatively little growth of the seminal vesicles before 32 days (Moore & Price, 1937) and that there is a marked increase in weight at the time of puberty, (Hooker, 1942). Since this might vary with different strains and under different laboratory conditions, it was necessary to determine the time at which the seminal vesicle weights for our rats were accelerated. Furthermore, we wanted to avoid undue delay for castration-atrophy and at the same time obtain uniform values for the controls. The data is summarized in Table 2. The mean seminal vesicle weight increased slowly from 4.7 mgm. in the 22 to 24 day old group to 6.3 mgm. for the 27 to 31 day old groups. From 32 to 50 days the rate of growth was markedly augmented as shown by the fact that seminal vesicle weights increased from 12.7 to 44.3 mgm.

TABLE 3. REGRESSION OF SEMINAL VESICLES OF RATS CASTRATED AT 50 DAYS AND RESPONSE TO 0.25 MG. OF TESTOSTERONE PROPIONATE

No. of Rats	Group	Interval from Castration to S. V. Removal (Days)	S. V. Weights (mgm.)	% Increase
12	Normal Controls		44.3	
3	Castrate Controls	30	32.9	
5	Castrates+T. P.	30	36.1†	10
5	Castrate Controls	57	18.1	
8	Castrates+T. P.	57	38.4†	110

† Testosterone propionate injected 72 hours prior to seminal vesicle removal.

Regression of Seminal Vesicles of Rats Castrated at 50 days of age and Response to 0.25 mgm. Testosterone Propionate

In this experiment, three groups of rats were used in order to determine whether the larger seminal vesicles of rats castrated at 50 days of age would regress to the pre-pubertal level and thus serve as a test object for control assay. The first group served as normal controls, the seminal vesicles being removed at 50 days. The second and third groups were castrated at 50 days of age and then killed after 30 and 57 days. Table 3 shows that while the seminal vesicles had markedly regressed at 30 and 57 days after castration, they had not approached the pre-pubertal control level. It was thought that even though the seminal vesicle weights were declining with an increased castration interval, the percentage response to a suitable dosage of testosterone propionate would be the same in each instance. Accordingly two groups of rats castrated at 50 days were given 0.25 mgm. of testosterone propionate at 30 and 57 days after castration respectively and seventy-two hours later the seminal vesicles were removed and weighed. The seminal vesicles of the 30 day group increased from 32.9 in the untreated to 36.1 mgm. in the treated, an increase of only 10

TABLE 4. EFFECT OF 1.0 MG. OF TESTOSTERONE PROPIONATE ON SEMINAL VESICLE WEIGHT AT VARIOUS INTERVALS AFTER CASTRATION ON 29TH DAY

No. of Rats	Castration Injection Interval days	Average Seminal Vesicle Weight (mgm.)	Standard Error	Number of Castrate Controls	Castrate Control Seminal Vesicle Weight (mgm.)
19	7–14	16.6	0.61	6	5.5
31	50–100	17.5	0.49	15	6.1
22	150–200	17.9	0.57	8	6.0
14	300–350	17.2	0.31	4	6.9

TABLE 5. INFLUENCE OF TIME AND DOSAGE ON THE SEMINAL VESICLE RESPONSE TO TESTOSTERONE PROPIONATE IN CASTRATE RATS

No. Animals	Dose T. P. Mgm.	Mean S.V. Wt.	S. E.	t	Sig.†
		(mgm.)			
		48 hour response			
16	0.10	13.8	0.40		
				2.94	+
13	0.25	16.4	0.79		
				0.49	−
10	0.50	16.9	0.66		
				2.13	+
16	1.00	18.7	0.53		
		72 hour response			
14	0.10	14.0	0.66		
				5.00	+
17	0.25	20.7	1.17		
				2.36	+
10	0.50	24.6	1.12		
				2.92	+
12	1.00	30.4	1.64		
		96 hour response			
10	0.10	12.2	0.54		
				4.25	+
10	0.25	19.3	1.58		
				1.54	−
10	0.50	23.4	2.15		
				5.09	+
11	1.00	37.7	1.82		
		120 hour response			
10	0.10	13.3	0.22		
				3.78	+
10	0.25	20.4	1.92		
				0.30	−
10	0.50	19.6	1.81		
				3.66	+
11	1.00	31.1	2.57		
		Controls			
33		6.1			

† Probability 0.05 or less considered significant

per cent; whereas the increase in the 57 day group was from 18.1 in the untreated to 38.4 mgm. in the treated, or an increase of slightly over 100 per cent. Thus, in order to get constant results in case of rats castrated at 50 days it is apparently necessary to wait for full regression before using them for assay purposes.

Effect of 1.0 mgm. of Testosterone Propionate on Seminal Vesical Weight at Various Intervals after Castration on 29th Day

Since it would be advantageous to be able to maintain a stock supply of castrate rats which would not have to be used at a specified interval after castration, a series of experiments was carried out to determine whether the seminal vesicles of rats castrated for various intervals would respond in the same manner to testosterone propionate.

One hundred and twenty-nine rats were castrated at ages varying from 27 to 31 days. These were divided into four groups of treated rats with corresponding controls. All 4 groups of treated rats received a single dose of 1 mgm. of testosterone propionate. Group 1 was injected 7 to 14 days after castration; group II, 50 to 100 days; group III, 150 to 200 days; group IV, 300 to 350 days after castration. Forty-eight hours after the injection the seminal vesicles were removed and weighed. The mean seminal vesicle weight range, as indicated in Table 5, was from 16.6 mgm. for those castrated 7 to 14 days, to 17.9 mgm. for those castrated 150 to 200 days. Statistically there was no significant difference getween any two groups and thus it is evident that the response to 1.0 mgm. of testosterone propionate was not influenced by the castration interval when the rats have been castrated at 27 to 31 days of age.

Influence of Time and Dosage on the Seminal Vesicle Response to Testosterone Propionate in Castrate Rats

Since we have been able to show that the extension of time from forty-eight to seventy-two hours in juvenile rats resulted in further growth of the seminal vesicles the present study was carried out to determine the optimum time for removal of seminal vesicles after treatment in castrate rats.

One hundred and ninety rats, castrated at 27 to 31 days of age were divided into four groups, in which different time intervals were selected for the removal of the seminal vesicles. In each group a dose-response curve was established. The doses used and the weights of the seminal vesicles are presented in Table 5.

The maximum increase in seminal vesicle weight was obtained after seventy-two hours for doses of 0.1, 0.25 and 0.5 mgm., while for 1.0 mgm. the maximum increase was not obtained until ninety-six hours. As shown in Figure 1, there was a slight decrease in seminal vesicle weight after ninety-six and one-hundred and twenty hours for the low doses, but for the 1.0 mgm. doses there was a marked regression

at one-hundred and twenty hours. The differences in seminal vesicle response to adjacent doses were smallest at forty-eight hours, became greater at seventy-two and ninety-six hours, and were again reduced by the end of one-hundred and twenty hours. Although the standard errors were smallest for the groups injected forty-eight hours, the difference in response to 0.25 mgm. and 0.50 mgm. was not statistically significant, as indicated by the value of "t" in Table 5.

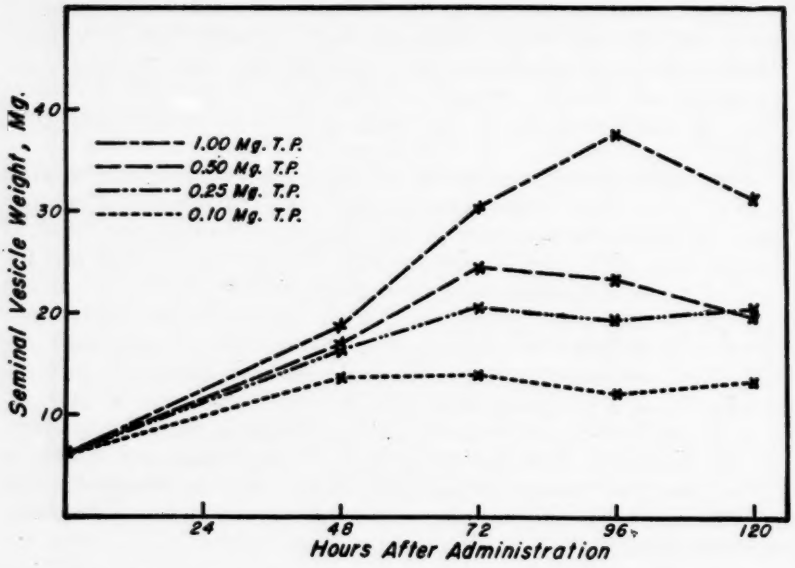

FIG. 1. The response of seminal vesicles in castrate male rats to various doses of testosterone propionate at 48, 72, 96 and 120 hours. The average seminal vesicle weight of castrate controls was 6.1 mgm.

At seventy-two hours, the differences between all adjacent groups were statistically significant, even though the standard errors were greater than those for the forty-eight hour group. At ninety-six hours the difference in response to 0.25 mgm. and 0.50 mgm. was 4.1 mgm. as compared with 3.9 for the same doses at seventy-two hours. However, the variability had increased to such an extent that the difference at ninety-six hours was no longer statistically significant. At one-hundred and twenty hours there was still no significant difference in the responses to these doses.

DISCUSSION

It is clearly evident from the data presented here that the measured response of the seminal vesicles to testosterone propionate depends to a large extent on the time of removal of the glands after injection. The time for maximum growth of the seminal vesicles is a

function of the dose and therefore the maximum response must be determined for each dose level for once this maximum has been reached, atrophy soon occurs. Greene & Burrill (1941) used a forty-eight hour interval for doses ranging from 0.005 mgm. to 0.25 mgm. which was probably the optimum time for those doses. Our results indicate that for doses of 0.25 mgm. to 1.0 mgm. seventy-two hours is the optimum time for removal of the seminal vesicles. This was well illustrated in experiments with colchicine, not included in this series but to be published later, that beyond seventy-two hours there is a definite regression of the seminal vesicles histologically.

The juvenile rat gave fairly satisfactory results in our laboratory but had the disadvantage that a very large colony of rats had to be maintained in order to obtain the large number of rats of the required age and weight range for a comparison between standard and unknown solutions of testosterone propionate. The use of castrate male rats circumvented this disadvantage. A sufficient number of test animals could be maintained since the response did not depend on the length of castration (Table 4). It was important, however, to determine the optimum age for castration since we have shown that once growth of the seminal vesicles has occurred atrophy is a slow process. Response of the seminal vesicles to testosterone propionate before atrophy has been complete is extremely variable (Table 3).

It has been generally assumed that the daily injection of male hormones is a better test for androgenic activity than the single injection method. We believe it is more desirable to measure the full effect of a single dose than the complex summation of multiple doses over widely different time intervals. Leathem (1945) has shown that the per cent increase in weight of seminal vesicles over controls for 0.25 mgm. of testosterone propionate was the same whether it was given in a single injection or in divided doses. Whether for higher doses this same relationship holds true has not been established but experience in this laboratory with other steroids suggest an additive effect. Our single injection technique for castrate rats simplifies the daily injection routine and yields quantitive results.

The response of seminal vesicles as shown in Figure 1, illustrate again the importance of taking into account the time interval as well as the dose. This relationship has too often been neglected in endocrine research.

SUMMARY

In 20 to 22 day old rats doses of testosterone propionate 0.25 and 1.00 mgm. produced a greater effect on seminal vesicle weights after seventy-two hours than after forty-eight hours and the responses to 0.10, 0.25 and 1.00 mgm. differed more significantly after the longer time interval. In normal rats the seminal vesicles showed little growth before 32 days, after which the rate of growth was accelerated. Rats castrated at 50 days were unsuitable for androgen assay because

of slow regression of seminal vesicles and inconstant seminal vesicle response to testosterone propionate at different intervals after castration. The seminal vesicles of rats castrated at 27 to 31 days respond in the same manner to a 1.0 mgm. dose of testosterone propionate at any time from 7 to 350 days after castration. The maximum effect on seminal vesicle growth in castrate rats was obtained seventy-two hours after injection with single doses of 0.1, 0.25 and 0.50 mgm. of testosterone propionate. With a 1.00 mgm. dose the maximum effect was obtained in ninety-six hours. At seventy-two hours after injection the differences in seminal vesicle response in castrate rats to doses of testosterone propionate ranging from 0.10 to 1.00 mgm. were more significant than at forty-eight, ninety-six or one-hundred and twenty hours.

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THE USE OF CASTRATE MALE RATS FOR THE ASSAY OF TESTOSTERONE PROPIONATE

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INTRODUCTION

IN A PREVIOUS COMMUNICATION Hays & Mathieson (1945) have (1) demonstrated that it is more convenient and leads to equally satisfactory results when castrate male rats are used instead of immature rats for assay of testosterone propionate; (2) shown that rats castrated at 26 to 29 days of age can be used for assay 14 to 300 days after castration; (3) obtained data which shows that seventy-two hours after the injection of testosterone propionate the difference in seminal vesicle response to doses ranging from 0.1 mgm. to 1.0 mgm. are more significant than at forty-eight, ninety-six or one-hundred and twenty hours. As a result of these experiments we have developed a method of assay for testosterone propionate using the new experimental design proposed by Bliss (1945) for determining the relative potency of drugs. This method together with detailed results of a representative assay is presented here.

METHODS

Rats of Wistar Strain were castrated between the ages of 26 to 29 days and weighing 40 to 75 grams. At least 14 days were allowed to elapse before using in any of the assays. In our colony the seminal vesicle weights at 29 days averaged 6.1 mgm. and showed little regression after 14 days castration. The technique for castration has been described many times and therefore is not reported here.

Two concentrations of testosterone propionate were prepared by diluting a standard 10 mgm. per cc. ampule with sesame oil so that the final concentration of the solutions to be tested contained 2.0 and 8.0 mgm. per cc. respectively. Unknown solutions corresponding to the standard solutions were prepared in the same way. When crystalline material was used it was first dissolved in ether and sesame oil added to half the volume. The ether was then removed under vacuum and the solution made up to final volume with sesame oil.

The solution to be tested was injected subcutaneously with a sterile syringe and needle. A twenty-four gauge needle was found preferable since it could be withdrawn easily and was less apt to allow leakage of material. The volume of oil was 0.1 cc. for all doses.

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Experimental Design.—The method outlined by Bliss (1945) was used in these experiments wherein a comparison was made of an unknown (U) against a standard (S) at two concentration levels such that the dilution ratio $U_2:U_1=S_2:S_1$. The rats were divided into four groups of eight each (Table 1). Group I was injected with 0.8 mgm. unknown; Group II with 0.2 mgm. unknown; Group III with 0.8 mgm. standard, and Group IV with 0.2 mgm. standard. In each assay the group (a) rats, i.e., a_1, a_2, a_3, a_4, were castrated for the same number of weeks, all of the group (b) rats for the same number of weeks but not necessarily for the same period as the group (a) rats. Likewise each group of rats (c) through (h) had similar periods of castration within groups. The distribution of rats and the subsequent mathematical treatment of results eliminated the variable which might have resulted from using rats castrated for different intervals or using rats of widely differing body weights.

TABLE 1. EXPERIMENTAL DESIGN FOR DISTRIBUTION OF TEST ANIMALS

	U_2	U_1	S_2	S_1
(*a*)	a_1	a_2	a_3	a_4
(*b*)	b_1	b_2	b_3	b_4
(*c*)	c_1	c_2	c_3	c_4
(*d*)	d_1	d_2	d_3	d_4
(*e*)	e_1	e_2	e_3	e_4
(*f*)	f_1	f_2	f_3	f_4
(*g*)	g_1	g_2	g_3	g_4
(*h*)	h_1	h_2	h_3	h_4

U_1 U_2 and S_1 and S_2 represent two corresponding doses of unknown and standard.

Removal of Seminal Vesicles. Seventy-two hours after the injection of test solutions, the rats were killed with a quick blow on the head. The ventral surface was opened to expose the male accessory organs and bladder. It was usually desirable to cut the body wall laterally so that the two flaps could be laid back, thereby allowing easier access to the seminal vesicles. With a pair of forceps and curved iridectomy scissors, each of the vas deferens was cut and then the seminal vesicles and prostate were removed by incising at a point near the base of the bladder. The tissue was dipped in physiological saline and placed on a cork board under a dissecting microscope. The coagulating glands were teased from the seminal vesicles and the latter incised at a point nearest the ejaculatory ducts. They were again immersed in saline, dried on blotting paper for a few seconds and weighed on a Roller-Smith balance.

RESULTS

The results are summarized in Table 2. The values in groups I to IV represent the individual seminal vesicle weights produced by the standard and unknown testosterone propionate. In U_1 and S_1 it will be observed that the individual variation was much greater than for U_2 and S_2. In the preceding paper (Hays and Mathieson 1945) the dose response curve which was established for testosterone propionate showed that with 0.1 mgm. the maximum response was at forty-eight hours while with 0.25 mgm. it was at seventy-two hours. It is possible that the large deviation in the response to 0.20 mgm. dose was due to some regression at seventy-two hours.

Calculation of Results.—The figures in Table 2, columns D_1, D_2, D_3 and D_4 represent four sets of differences for each of the eight groups (a–h). These differences are used in computing y_1, y_2 and y_3. The values for $y_1 = D_1 + D_2$, show the differences between standard and unknown; while $y_2 = D_3 + D_4$, represents the difference between the two doses. The differences $y_3 = D_1 - D_2$ or $D_3 - D_4$ are used in testing whether the dose response curves are parallel. To calculate the potency of the unknown, determine the logarithm of the relative potency M' by the formula:

TABLE 2. RESPONSE OF SEMINAL VESICLES TO STANDARD AND UNKNOWN TESTOSTERONE PROPIONATE AND CALCULATION OF RESULTS

Group No.	Group I U_3	Group II U_1	Group III S_2	Group IV S_1	$D_1 = U_3 - S_2$	$D_2 = U_1 - S_1$	$D_3 = U_3 - U_1$	$D_4 = S_2 - S_1$	$y_1 = D_1 + D_2$	$y_2 = D_3 + D_4$	$y_3 = D_1 - D_2 = D_3 - D_4$
a	32.1	23.9	29.5	13.8	2.6	10.1	8.2	15.7	12.7	23.9	−7.5
b	32.6	23.6	33.3	23.4	−0.7	0.2	9.0	9.9	−0.5	18.9	−0.9
c	27.4	17.0	23.0	17.1	4.4	−0.1	10.4	5.9	4.3	16.3	4.5
d	28.4	24.7	29.6	15.9	−1.2	8.8	3.7	13.7	7.6	17.4	−10.0
e	35.9	23.5	32.3	25.1	3.6	−1.6	12.4	7.2	2.0	19.6	5.2
f	27.0	10.9	35.1	21.3	−8.1	−10.4	16.1	13.8	−18.5	29.9	2.3
g	29.3	17.2	27.0	18.0	2.3	−0.8	12.1	9.0	1.5	21.1	3.1
h	37.1	16.1	29.2	24.4	7.9	−8.3	21.0	4.8	−0.4	25.8	16.2
									$T_1 = 8.7$	$T_2 = 172.9$	$T_3 = 12.9$

$$M' = \frac{IT_1}{T_2} = \frac{0.6021 \times 8.7}{172.9} = 0.0303$$

where

$$I = \log\left(\frac{\text{larger dose}}{\text{smaller dose}}\right).$$

The estimated potency of the unknown in terms of mgm./cc. is found by determining the antilog of the estimated log potency M.

$$M = \log (\text{assumed potency}) + M^1$$

$$M = 1.000 + 0.0303 = 1.0303$$

$$\text{Antilog of } M = 10.72 \text{ mgm./cc.}$$

Since the accuracy of this method is dependent upon the standard error and on the assumption that the dose response curves are parallel we have followed the procedure used by Bliss (1945) to determine these points.

(1) Slope of dose-response curve

$$b = \frac{T_2}{2IN} = \frac{172.9}{2 \times 0.6021 \times 8} = 17.95$$

where N = number of groups of rats.

(2) Standard deviation of a single response

$$s = \sqrt{\frac{S(y^2) - (T_1^2 + T_2^2 + T_3^2)/N}{12(N-1)}}$$

$$s = \sqrt{\frac{4954.03 - 3767.06}{84}} = 3.7590$$

where y refers to all individual values of y_1, y_2, y_3, and $S(y_2)$ is the sum of their squares.

(3) Standard deviation in terms of log-dose.

$$\lambda = \frac{s}{b} = \frac{3.7590}{17.95} = 0.2094.$$

(4) The Standard Error of M or M^1 in log units is equal to:

$$S_M = \lambda \sqrt{\frac{1}{N}\left(1 + \frac{T_1^2}{T_2^2}\right)}$$

$$= 0.2094 \sqrt{\frac{1}{8}\left(1 + \frac{75.69}{29894.41}\right)} = 0.0741.$$

(5) Standard error in original units is

$$\text{S.E.} = 2.30\, S_M(\text{antilog } M)$$

$$\text{S.E.} = 2.30 \times 0.0741 \times 10.72 = 1.83$$

Thus it will be seen that for 4 groups of 8 rats each, the estimated potency of a 10.0 mgm./cc. sample of testosterone propionate was 10.72 ± S.E. 1.83.

The type of analysis can be used only where the dose-response curves for standard and unknown are parallel. This assumption may be tested by the "t" test where

$$t = \frac{T_3}{2s\sqrt{N}} = \frac{12.9}{2 \times 3.7590 \times 2.83} = 0.61.$$

The observed value when referred to a "t" distribution table gave a probability of 0.55 and therefore exhibited no divergence from parallelism.

A second assay carried out on the same sample of material in exactly the same manner as the above mentioned gave a potency of 9.26 mgm./cc. ± S.E. 1.74, again with no divergence from parallelism.

SUMMARY

The response of seminal vesicles in castrate male rats to testosterone propionate has been used as a criteria of androgenic activity. The assay was designed so that a comparison could be made between

standard and unknown solutions of testosterone propionate at two dose levels. Statistical treatment of the data yields an estimate of the potency of the unknown together with its standard error. Two assays carried out simultaneously on a 10 mgm./cc. sample of testosterone propionate gave values of 10.72 mgm. and 9.26 mgm. respectively.

ACKNOWLEDGMENTS

We wish to express our appreciation to Dr. E. Oppenheimer for his valuable criticisms and suggestions throughout the investigation and to Mr. Joseph Lein for his advice on the statistical treatment of the data.

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THE DURATION OF ACTION OF SINGLE DOSES OF THYROID HORMONE[1]

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REPORTS on the duration of action of thyroid compounds vary considerably. As early as 1904, Magnus-Levy reported that the effects on the basal metabolic rate of thyroid feeding in myxedematous patients lasted 80 days. Kendall (1917), probably the first to inject pure thyroxin into animals, compared the effects of small daily doses and a single large dose. A goat receiving 9 mg. daily was dead at the end of 11 days; the single injection of 150 mg. in another goat produced changes in the electrocardiographic tracings which returned to normal in 2 or 3 days. In contrast to these results, Kendall (1923) reported that a single dose of thyroxin in a myxedematous patient would exert its physiologic effect for 5 to 6 weeks.

Boothby, Sandiford, Sandiford, and Slosse (1925) showed that the rate of decline of the basal metabolism following withdrawal of thyroid administration in myxedematous patients was a straight line, when plotted on semilogarithmic paper. They reported that a total of 3.9 gm. of desiccated thyroid given in 3 days would exert its effect on the basal metabolic rate for 60 days, while 11 mg. thyroxin, given in 6 days, would last for 40 days. Boothby and Sandiford previously reported (1924) that the effect of a single intravenous injection of 16 mg. thyroxin in a myxedematous subject lasted 30 to 70 days.

According to Gaddum (1929), the duration of effect depends on the method of administration. Thyroxin in alkaline solution, given intravenously, affects the basal oxygen consumption for only 3 days; if an alkaline solution of thyroxin is injected subcutaneously, the basal oxygen consumption will not return to normal for 3 weeks.

Salter, Lerman and Means (1933) and Thompson, Thompson, Brailey and Cohen (1929) have compared the effects of single doses of various thyroid preparations, using varied routes of administration. According to Salter, Lerman and Means, thyroxin polypeptide will exert the same effect, whether given orally or intravenously. Given intravenously, 13 mg. of the polypeptide will exert an effect similar to

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that of 10 mg. of pure thyroxin. The effects of a single dose were still detectable at the end of 90 days.

Thompson compared the effects of pure thyroxin, the monosodium salt of thyroxin, thyroxin polypeptide, and desiccated hog thyroid. He found that pure thyroxin, given in alkaline solution by mouth, was the most effective, lasting 33 to 95 days; a thyroxin suspension by mouth or duodenal tube caused no definite change in the basal metabolic rate; intravenous thyroxin lasted 29 to 80 days. The monosodium salt exerted an effect for 25 to 52 days, the polypeptide for 20 to 54 days, desiccated thyroid for 33 to 69 days. Thompson states that "in terms of basal metabolic rate, the effect of the injection of 10 mg. of thyroxin in myxedema is completed in 90 days, but in terms of general well-being, the effect lasts for some time longer—probably several months" (Thompson, 1929, p. 458).

Plummer (1921) states that "after the administration of a single dose of thyroxin sufficient to bring the basal metabolism to normal, the physiologic status of a thyroidless patient becomes normal in 10 to 12 days, remains approximately normal for 10 days, and returns to the pre-existing state in from 5 to 7 weeks" (p. 243).

Recently, experiments have been conducted on the implantation of thyroxin pellets subcutaneously. Wokes (1941), using guinea pigs, compared the effects of orally administered thyroid and subcutaneous thyroxin pellets. He reported that 100 to 200 mg. thyroid affected the basal metabolism for about 5 days, while a 25 mg. pellet of thyroxin lasted 20 to 30 days. Parkes (1924) implanted 25 to 50 mg. pellets of thyroxin in rats and claimed that there was no weighable absorption of the pellets over a period of 207 days. Rowlands (1942) implanted 40 mg. tablets in thyroidectomized rats. Although enough thyroxin was absorbed to maintain normal growth, his calculations showed that the rate of absorption was so slow that such a tablet would last a rat 40 to 50 years.

The concentration of serum cholesterol was used as a criterion of thyroid function by Wilkins, Fleischmann, and Block (1941). They reported that a single intramuscular injection of 2 to 5 mg. thyroxin in hypothyroid children caused a decrease in serum cholesterol lasting from 32 to 57 days (in one exceptional case, 71 days), while in normal children the cholesterol level was affected for only 6 to 13 days.

The discovery of the ability of thiourea derivatives to inhibit thyroid function (MacKenzie and MacKenzie, 1943; Astwood, Sullivan, Bissell and Tyslowitz, 1943) suggested a new method of approach to the problem of the duration of thyroxin action. Using thyroid weights and thyroid iodine as criteria, it has been shown that within 48 hours after the beginning of thiouracil administration, the hormone within the thyroid gland is markedly reduced, and compensatory hyperplasia begins (Astwood and Bissell, 1944). Therefore, if thyroxin were administered to animals previously rendered incapable of synthesizing thyroid hormone, a beginning increase in

thyroid weight would indicate that the quantity of thyroid hormone remaining in the body had fallen below the normal level. This would provide a measure of the rate of destruction or elimination of artificially administered thyroid hormone.

EXPERIMENTAL

Young rats of the Long-Evans strain, weighing from 50 to 80 gm., were used. Thiouracil treatment was initiated by the subcutaneous injection of 25 mg. thiouracil.[2] From this time until the end of the experiment, thiouracil

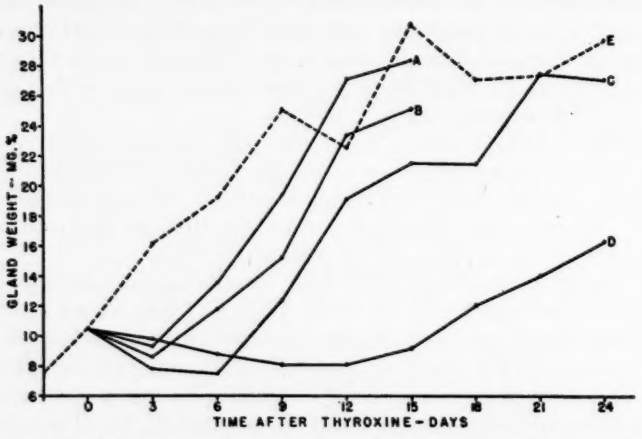

FIG. 1. Effects of a single injection of thyroxin on the rate of enlargement of the thyroid glands of thiouracil-treated rats. A=10 μg. (solution); B=100 μg. (solution); C=1 mg. (solution); D=1 mg. (suspension); E=control animals, receiving thiouracil alone. The initial point indicates the gland weight at the beginning of thiouracil administration and 0 the time when thyroxin was injected. Each point represents the average of 5 or more animals.

was given as a 0.1% solution in the drinking water. Forty-eight hours after the beginning of thiouracil treatment, a single dose of thyroxin[3] or desiccated thyroid was given; at stated intervals thereafter, animals were killed with chloroform, the thyroid glands dissected out and weighed immediately.

The thyroxin was either dissolved in distilled water with the addition of just enough NaOH to effect solution, or the finely ground crystals were suspended in 0.2% agar solution. The desiccated thyroid was suspended in distilled water and administered by stomach tube.

RESULTS

The results of injecting varying quantities of thyroxin subcutaneously, in alkaline solution or in suspension, are given in Figure 1. It will be seen that after the injection of thyroxin, the thyroid

[2] Thiouracil was supplied by Dr. B. W. Carey of the Lederle Laboratories, Inc., Pearl River, New York.

[3] Thyroxin (Squibb) was supplied by Dr. G. A. Harrop.

weight was depressed for a short period, and then began to increase, eventually paralleling the growth rate of the glands of animals maintained on thiouracil alone. The point at which the weights began to increase has been used in estimating the duration of action of the administered thyroxin. Ten μg. and 100 μg. lasted 3 days; 1 mg. (in solution), 6 days. In comparison with these values, 1 mg. injected as a suspension lasted 12 days. It will also be noted that the rate of thyroid growth following the administration of the thyroxin suspen-

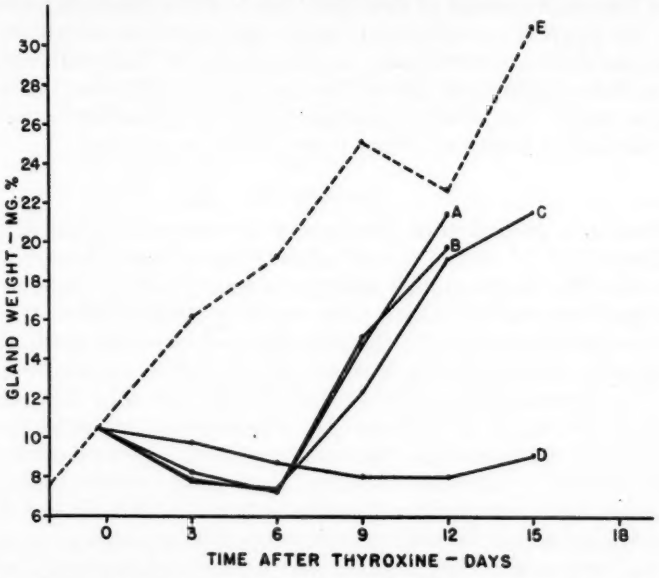

FIG. 2. A comparison of the effect of single doses of thyroid hormone, given by various routes to thiouracil-treated rats. A=1 mg. thyroxin in solution intravenously; B=500 mg. desiccated thyroid by stomach tube; C=1 mg. thyroxin in solution subcutaneously; D=1 mg. thyroxin suspension subcutaneously; E=control animals, receiving thiouracil alone. The initial point indicates the gland weight at the beginning of thiouracil treatment and 0 the time of thyroid hormone administration. Each point represents the average of 5 or more animals.

sion does not parallel that of thyroxin in solution, indicating, perhaps, a small residual deposit of the thyroxin crystals.

It was thought that it would be of interest to compare different routes of administration. In one group of animals, 1 mg. thyroxin was injected into the tail vein. In another group, 500 mg. desiccated thyroid, which was calculated to be approximately equivalent to 1 mg. pure thyroxin, was given by stomach tube. The results of these experiments are compared with the effects of 1 mg. thyroxin subcutaneously in Figure 2. It will be seen that the route of administration had no effect on the duration of action of these doses of thyroid hormone administered in solution.

An attempt was made to study the effect of very large doses of thyroxin given intraperitoneally. It was found that 100 gm. rats could not tolerate more than 50 mg. by this route; in fact, even this dose proved fatal in a large number of animals. Those animals which died within 48 hours of the injection exhibited, at autopsy, an acute peritonitis, probably due to the high concentration of alkali necessary to affect the solution of the thyroxin. Deposits of precipitated thyroxin were found scattered throughout the peritoneal cavity. From the small amount of data available in this experiment, it seemed that the thyroxin continued to exert an effect for about 30 days. Thyroxin deposits were found in those killed at 6 and 15 days, but not in those killed at 30 days. The precipitated hormone, by forming a depot which was slowly absorbed, made it impossible to determine the duration of action of a very large dose as a solution.

DISCUSSION

There is a great deal of discrepancy between the results of various workers in regard to the rate of elimination of administered thyroid hormone. The above results indicate a much shorter time of action than that reported by others, even for large doses. Part of the variation could be explained by the fact that a few of the earlier experiments were carried out on normal subjects, while the majority were done on hypothyroid patients. Both Thompson and Wilkins have noted a difference in response to administered thyroxin between normal and myxedematous patients, reporting a greater effect in the latter. Winkler, Lavietes, Robbins and Man reported that non-myxedamatous patients can tolerate as much as 6 grains of thyroid daily, while there is no tolerance developed in myxedema, even after years of treatment. They suggest that normal subjects may have the ability (lacking in myxedema) to inactivate thyroxin, and propose the thyroid gland itself as the agent.

The use of basal metabolic rate determinations as a criterion of the duration of action of a given dose of thyroxin may provide misleading information. It is conceivable that even after the thyroxin has been completely eliminated from the body, the basal metabolic rate would continue to fall as a result of secondary changes which constitute the full picture of myxedema. Changes in the metabolic rate would then indicate not only the length of time that the thyroxin was active, but also the time that it takes the patient to revert to his pre-treatment level. The method described in this paper appears to offer a more accurate determination. Since thiouracil inhibits the formation of hormone by the thyroid gland and causes a compensatory hyperplasia of the gland when the store of thyroxin is exhausted, any increase in the thyroid weight subsequent to the injection of thyroxin in hypothyroid animals indicates that an insufficient proportion of the administered hormone still remains available in the body.

The physical state of the thyroxin influences the duration of its

action. Figure 1 shows that a suspension of finely ground thyroxin crystals will last approximately twice as long as an equivalent amount given in solution by the same route. The above results are not in disagreement with those of Parkes or Rowland, who report that 40 to 50 mg. of thyroxin last for an exceedingly long period. Fifty mg. injected intraperitoneally was found to be precipitated within a few hours. In animals from this group, killed at the end of 30 days, the precipitated thyroxin had entirely disappeared. This more rapid absorption of solid thyroxin is probably attributable to the large surface of the finely precipitated material.

It is possible that there is a difference in the rate at which thyroid hormone is degraded or excreted, depending upon whether physiological or excessive quantities are present in the body.

SUMMARY

The duration of action of single doses of thyroxin and desiccated thyroid in rats pretreated with thiouracil has been studied. Thyroxin injected subcutaneously or intravenously in solution exhibited effects for as long as equivalent doses of thyroid powder by mouth. The duration of effect increased with the size of the dose. Suspended thyroxin crystals exerted an effect longer than thyroxin in solution. Single injections up to 50 mg. intraperitoneally were completely metabolized within a month. It is believed that the methods used in these experiments give a more accurate determination of the duration of action of thyroid hormone than is given by basal metabolic rate determinations.

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QUANTITATIVE STUDIES ON RESPONSE OF SEX CHARACTERS OF DIFFERENTIATED RANA CLAMITANS LARVAE TO INJECTED ANDROGENS AND ESTROGENS[1]

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AMPHIBIANS have frequently served as the subjects of experiments designed to investigate the nature of sex differentiation. Various agents were employed successfully to effect extensive alterations (reviewed by Witschi, 1939; Humphrey, 1942), but it is only within relatively recent years that commercial hormone preparations in the form of tissue extracts or synthetic compounds have opened up new lines of inquiry. The increasing degree of purity of these preparations has made possible more exact and quantitative studies of their effects on sex development. As early as 1930, Witschi and co-workers injected estrogenic hormones extracted from urine of pregnant women into the body cavities of Rana clamitans larvae, with the result that "although fairly large quantities were administered no effect beyond unspecific degeneration in the ovaries could be detected" (Foote and Witschi, 1939). In 1936, Padoa made the surprising report that he had obtained a so-called "paradoxical" masculinization of the gonads of Rana esculenta tadpoles with Cristallovar, a commercial extract of pregnancy urine. One year later both Gallien and Witschi and Crown secured a reversal of frog females to males with a pure preparation of testosterone. Similar findings were reported by others (Gallien, 1938; Foote, 1938; Foote and Witschi, 1939; Puckett, 1940; Witschi, 1942) on different species of Rana. Feminizing effects of estrogens on genetical male anurans have been demonstrated by Witschi and Crown (1937), Foote (1938), Gallien (1938), Puckett (1940), Dantschakoff (1941, cited in Witschi, 1942), and Witschi (1942) in several species of frogs and tree frogs.

Most of the above experiments had been conducted on young larvae whose genetical sex constitution was not known and which were at a sexually indifferent stage when treatments were begun. A series was therefore started in 1938 in which older Rana clamitans

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larvae of known sex with well-developed gonads were used. A preliminary paper (Foote and Witschi, 1939) and two abstracted reports of further quantitative studies have appeared (Witschi, Foote, and Mintz, 1942; Mintz, 1944).

MATERIALS AND METHODS

Rana clamitans tadpoles were collected locally and treatments started shortly thereafter. The animals of series I (collected in August) and series II

TABLE 1. SUMMARY OF TYPES OF TREATMENT ADMINISTERED

Series	Treatment	Dose	No. of larvae	Original sex	Gonadotropins added[4]	Dose	No. of larvae
Ia.	Testosterone propionate[1]	.250 mg.	39	♀			
b.	Estrogens[2]	.032–.320 mg.	26	♂			
c.	Controls, Oil	.050 ml.	9	5 ♀, 4 ♂			
d.	Controls, uninjected	—	15	8 ♀, 7 ♂			
IIa.	Testosterone propionate[1]	.050 mg.	9	♀	B.P.	5 mg./wk.	3
b.	Testosterone propionate[1]	.250 mg.	49	♀	B.P.	5 mg./wk.	7
					G.	.05 ml./wk.	4
					B.P.+G.	5 mg.+.05 ml./wk.	6
					*1G.+C.	.05 ml.+1 mg.	2
c.	Testosterone propionate[1]	.250 mg.	23	♂	B.P.	5 mg./wk.	6
					G.	.05 ml./wk.	1
d.	Estradiol dipropionate[3]	.032 mg.	8	♂	B.P.	5 mg./wk.	3
e.	Estradiol dipropionate[3]	.320 mg.	45	♂	B.P.	5 mg./wk.	10
					G.	.05 ml./wk.	5
					B.P.+G.	5 mg.+.05 ml./wk.	5
					*1G.+C.	.05 ml.+1 mg.	1
f.	Estradiol dipropionate[3]	.320 mg.	22	♀	B.P.	5 mg./wk.	5
					*1G.+C.	.05 ml.+1 mg.	1
g.	Controls, Oil	.050 ml.	13	6 ♀, 7 ♂			
h.	Controls, uninjected	—	13	7 ♀, 6 ♂	*1G.+C.	.05 ml.+1 mg.	1 ♀, 1 ♂
IIIa.	Testosterone propionate[1]	.025 mg.	17	♀			
b.	Testosterone propionate[1]	.050 mg.	32	♀			
c.	Controls, uninjected	—	16	♀			

[1] Perandren, Ciba
[2] Estradiol 3-benzoate-17-n-butyrate, followed by estradiol (Ovocylin, Ciba)
[3] Di-ovocylin, Ciba
[4] B.P.: Beef pituitary powder; 5 mg.≏.1 R.U.
G.: Pregnant mare serum (Gonadin, Cutter); .05 ml.≏6.25 mg.≏5 R.U.
C.: Sheep pituitary extract (Ciba); 1 mg.≏5 R.U.
* 1G.+C.: In these four instances, only one injection of each was given.

(collected in December) measured 7–9 cm. total length; those of series III (collected in December) measured 6–8 cm. Both males and females were used in the first two series. The sex of most of these animals was first ascertained by exploratory laparotomy. Some animals of unknown sex were also treated, but in subsequent examination there was with one possible exception no difficulty in determining the genetical sex. In the third series, all larvae were laparotomized and only females used. All injections were intraperitoneal. The steroids employed were dissolved in .05 ml. of sesame or corn oil per injection. A number of animals from each series served as controls,—either un-

treated or injected with oil alone. Some animals (in series II) received gonadotropin injections in addition to the steroids. All injections were made weekly, with the exception of those in series I, in which weekly and biweekly injections were alternated in some of the larvae, depending upon the apparent hardihood of the animals in reacting to the treatment. Table 1 summarizes the data on substances used and number of animals treated with each.

RESULTS

Hermaphrodism has never been reported as a normal occurrence in this species, nor are there any hermaphrodites among the controls of our experimental material. Control animals injected with oil show occasional degeneration of gonad tissue. Such degeneration is, however, unspecific, involving various parts of the gland indiscriminately. None of the sex hormones employed has any appreciable effect upon the onset or rate of metamorphosis of the experimental larvae as compared with controls. Mortality is in general increased by the injections, possibly owing to some toxicity of the hormones.

Injection of estradiol, estradiol 3-benzoate-17-n-butyrate, or estradiol dipropionate in concentrations of .032 to .320 mg. per week or biweekly over a maximum period of 34 weeks elicits no marked response in either males or females. Neither oviducts nor mesonephric ducts are affected, nor is the progress of ovogenesis or of spermatogenesis. Some degeneration of auxocytes is observed in many females; in no case has such degeneration proceeded very far. In males, however, there occurs a noticeable distention of the proximal part of the rete testis near the hilus. This trend in the direction of the female ovarial sac condition was cited (Foote and Witschi, 1939) as a reminder of the homology of the rete testis in the male and the ovarial sacs (follicular theca) arising from the rete ovarii in the female.

Administration of testosterone propionate to females causes a reversal of sex which is so complete that in every respect the conditions of the typical male become established within five months (Figs. 5 and 6). The ovaries shrink, and by the time the stage of a female-male sex intergrade is reached (as shown by microscopic examination), the gonads are more slender, less convoluted, slightly shorter, and have a smoother surface than control ovaries (see Fig. 1, Foote and Witschi, 1939 for the control condition). And by the time histological sections show these glands to be almost or fully male, their outward aspect also is much like that of control testes (for controls, see Fig. 2, Foote and Witschi, 1939), differing from them only in being of slightly larger size. Changes take place in a fairly uniform sequence in all animals. The course of events may be described as follows:

1. Beginning degeneration of the cortex, starting at the periphery and attacking first the young leptotene and synaptene ovocytes.
2. Progression of cortical degeneration, including auxocytes.

3. Pronounced or complete cortical degeneration, with the germinal epithelium left as peritoneum only.

4. Proliferation of the theca to form interstitial cells.

5. Proliferation and migration of mesonephric blastema cells down through the hilus; organization of the rete testis in the medulla.

6. Formation of vasa efferentia.

7. Marked decrease in size of sex glands.

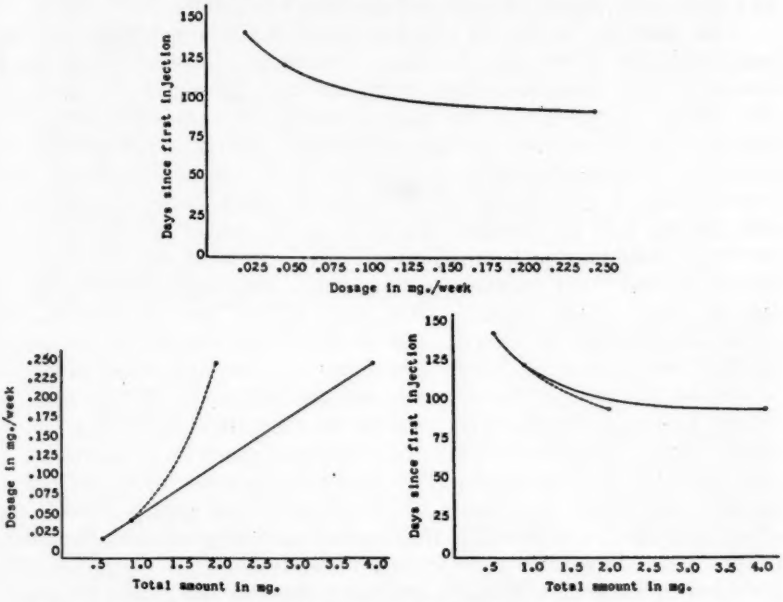


FIG. 1. Quantitative relationships. _____ Injected weekly; - - - injected alternately weekly and biweekly. Further explanations in the text.

8. Migration of gonia and their surrounding follicle cells from cortex to medulla.

9. Embedding of gonia in the rete cords (Fig. 3).

10. Expansion of the distal ends of the rete cords to form seminiferous tubules.

11. Spermatocyte formation.

12. Maturation; formation of spermatids.

13. Spermiogenesis (Figs. 4 and 5).

These changes overlap to a certain extent, especially with reference to cortical degeneration. After cytolysis of the auxocytes has become marked, the subsequent changes start. Some auxocytes in degenerating stages may still be present when the gonad is otherwise typically male (Fig. 4). In the case of animals killed in May, one finds that the normal rapid spring proliferation of young ovocytes in early

prematuration stages (Fig. 2) has been suppressed. Such animals have few young ovocytes when compared with their control sisters. Although the hormone first exerts its influence on young ovocytes, the gonia are not affected despite their proximity. It is these same gonia, originally destined to become ovogonia and eventually ova, which migrate into the interior in strands of two or three (sex cords) and take up their new definitive positions in the testis tubules, there to function as spermatogonia and give rise to sperms.

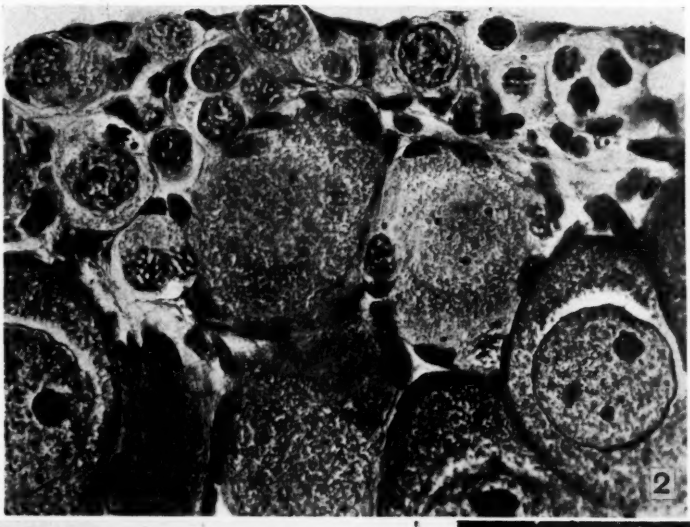
The decrease in size of the sex gland (7) is a gradual one. Its position in the above list of changes marks the time when the final size is attained. In each dosage group (.25 mg., .05 mg., and .025 mg.) the degree of change in the male direction increases steadily and regularly as the number of days of treatment increases. Migration of gonia (8) was decided upon as the decisive event which marks the transition to a type clearly resembling the male more than it does the female; this is therefore regarded as the stage of distinct sex reversal. After an injection period covering a definite number of days in each group, every animal is sex-reversed. This time element differs for the three dosage groups. Of a total of 146 females treated, 69 lived long enough to undergo sex reversal; the remaining animals, having been preserved before this stage was reached, show the less advanced changes. There are seven females (all in the .25 mg. group) which have undergone only cortical degeneration and some theca proliferation after treatment sufficiently prolonged to sex reverse all other animals in that group. The bodies as well as gonads of these animals are larger than those of all others in the group. It is concluded that they are probably neotenic larvae one year older than the others with which they were found. These females are therefore not included in Table 2, which is a summary chart of the gonad response and progressive sex inversion of the remaining 139 animals.

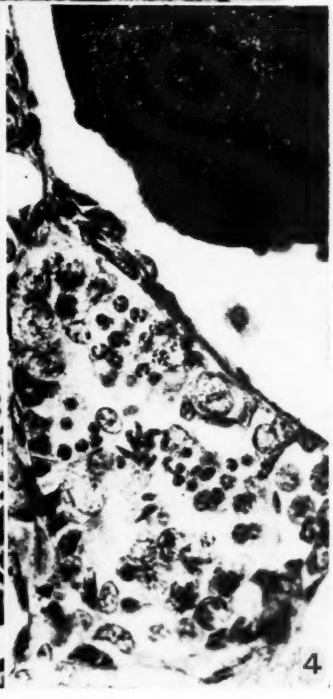
The first category (female) designates those that are still almost typically female, and includes animals listed as 1 and 2 in the sequence of changes previously described. From this point on, change 3 overlaps all other changes. Intergrade 1 represents event 4. Intergrade 2 includes 5, 6, and 7. Intergrade 3 marks sex reversal and is synonymous with 8 and 9. The last column (male) is reserved for animals in steps 10 through 13,—individuals typically or almost typically

FIG. 2. Cross section through ovary of a control female larva killed in May showing spring proliferation of young peripheral ovocytes. ×500.

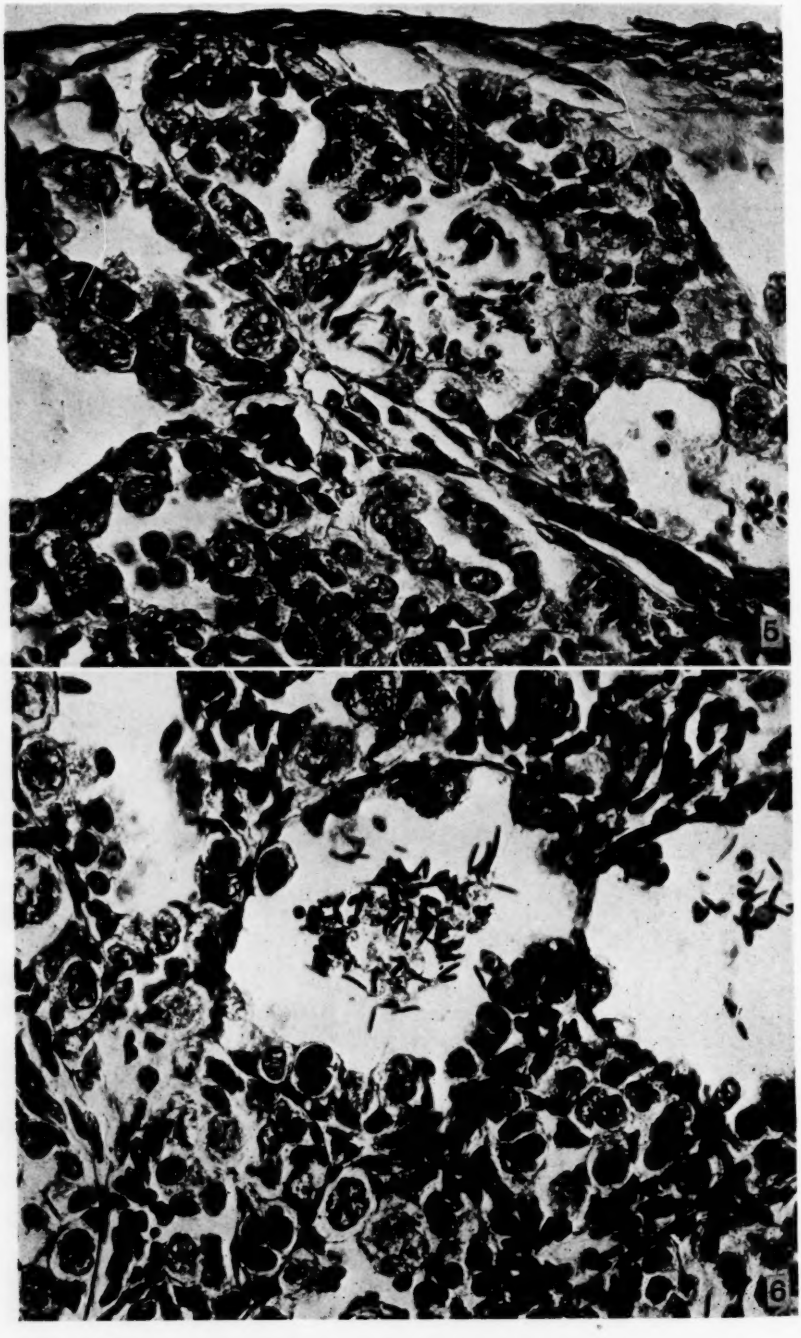
FIG. 3. Cross section through gonad of a female sex-reversed to male after 151 days' injection with a total of 1.1 mg. of testosterone propionate. Gonia have migrated from the cortex and are being embedded in the distal parts of the medullary cords. ×250.

FIG. 4. Cross section through gonad of a sex-reversed female killed in August and injected with a total of 8.25 mg. of testosterone propionate over a period of 230 days, during which time the animal metamorphosed. The tubule contains spermatids and some sperms. Adjacent to it a part of a degenerated egg is visible. ×500.





FIGS. 2–4. See opposite page for description.



FIGS. 5–6. See opposite page for description.

male. Classification in any of these five groups denotes farthest advancement in inversion.

Sex reversal with weekly injections of testosterone propionate is obtained in 95 days with a total of 4 mg. (at .25 mg./wk.), in 123 days with a total of .9 mg. (at .05 mg./wk.), and in 144 days with a total

TABLE 2. PROGRESSIVE SEX INVERSION OF 139 FEMALE LARVAE INJECTED WITH TESTOSTERONE PROPIONATE

Duration of injections in days	Dosage in mg./wk.[1]	Total amount per larva in mg.	Number of animals and type of reaction[2]				
			Female	Intergrade 1	Intergrade 2	Intergrade 3	Male
8–15	.25	.250– .500	8	2			
18–29	.25	.500– .750	3	1	2		
33–37	.25	1.000–1.250	2	2			
38–51	.25	1.250–1.750	5	3			
59–71	.25	1.500–2.500	5	4	4		
73–95	.25	1.750–3.500	1	4	3		
95–137	.25	2.000–4.000				23	2
166–230	.25	6.000–8.250				3	4
12–71	.05	.100– .500	4		1		
73–115	.05	.550– .850	5	5	2		
123–148	.05	.900–1.050				5	
151–154	.05	1.100				9	10
55–108	.025	.200– .400	2	2			
144	.025	.525				1	
151–152	.025	.550				11	1
		Totals	35	23	12	52	17

[1] Or biweekly, in the case of some animals receiving the .25 mg. dosage
[2] See text (Results) for significance of categories

of .525 mg. (at .025 mg./wk.). When the .25 mg. dose is administered alternately weekly and biweekly, sex reversal still occurs after the same lapse of time (95 days), but the total amount of hormone used per larva is reduced to only 2 mg.

Of the 69 animals that were sex-reversed, only five were continued on the experiment until the time of year (July and August) when sperms started to appear in control males. Of these five, three actually had sperms. This proportion is similar to that found among the controls, where two out of four had sperms. Microscopically, it is impossible to distinguish between the germ cells of the experimental and control males. Some sex-reversed individuals killed at earlier times of the year have gonads with well-formed seminiferous tubules containing spermatocytes. It is likely that these would have become functional testes even without further injections.

FIG. 5. Another section through the sex gland of the same animal as in Fig. 4. Sperms are clearly visible in one of the tubules. ×500.

FIG. 6. Cross section through the testis of a control male which metamorphosed in the laboratory and was preserved in August at the end of the experiment. Note sperms. ×500.

The gonads of males injected with testosterone propionate show no change.

Limited observations on gonoducts indicate that in females as well as males receiving testosterone propionate the larval mesonephric duct is but little responsive to the male hormone. Some cases show a slight stimulation of the duct just before metamorphosis. In one male this stimulation has proceeded to the extent of formation of small seminal vesicles on the lower portions of the vasa deferentia. (Rana clamitans controls do not have seminal vesicles.) In control animals, the oviduct does not arise until just before metamorphosis, when it appears as a small inconspicuous duct in males as well as in females. Its formation in testosterone-injected experimentals is not affected in either sex.

Addition of one or more gonadotropic substances (beef pituitary, pregnant mare serum, sheep pituitary extract), even when given over 59 days in concentrations as recorded in Table 1, has no influence on degree or rate of change in animals receiving sex hormones. (These additions were noted in Foote and Witschi, 1939, but the results not described as the experiments had not yet been terminated.) Puckett (1939), however, obtained precocious development of the gonads of an "undifferentiated" race of Rana catesbiana tadpoles with injections of mammalian pituitary extracts, as well as acceleration of the development of sex-reversed gonads of larvae receiving both sex hormone and pituitary injections.

DISCUSSION

Relationships of the quantitative factors (dosage, time, and total amount of hormone) involved in sex transformation of females to males with testosterone propionate are recorded in graphic form in Figure 1.

These graphs appear to indicate that:

1. The time required for sex reversal increases as the dosage decreases.
2. It is probable that the minimal effective dose has not yet been reached.
3. At this minimum, the number of days required for sex reversal probably would not be much greater than 150.
4. Doses higher than .25 mg. probably would not shorten the time necessary for sex reversal much below 95 days.
5. The total amount of hormone required (injected weekly) increases linearly with the dose.
6. Biweekly injections considerably decrease the total amount of hormone necessary at a given dosage as compared with weekly injections.
7. Biweekly injections do not change the time necessary for sex reversal at a given dosage.

8. There is an inverse relationship between the total quantity of hormone and the number of days required for sex reversal (with weekly injections).

9. With weekly injections, the total amount of hormone required for sex reversal at the minimum number of days is close to 4 mg.

Biweekly injections are apparently much more economical than weekly ones, as they produce the same effect in the same amount of time with much less total hormone. This indicates a limited or slow utilization of the hormone by the animal.

The apparent mechanism whereby testosterone propionate effects a reversal of sex from female to male is by destruction of the cortex of the sex gland. Subsequent hypertrophy of the medulla is then mainly a secondary compensatory phenomenon. Cortical degeneration is followed by the inception of medullary growth; an initial or direct medullary stimulation does not occur (nor is the medulla of males stimulated). Presumably the drastic decrease in cortical tissue would remove from the scene of action no only corticin + (inducing female development of primary gonia in the cortex), but also corticin − (inhibiting medullary growth). The medulla is then relatively free to grow under the influence of its own inductor substances (medullarin + and −) and without the previous depressing effect genetically imposed upon it through the agency of the cortex. The deleterious action of the male sex hormone on the cortex in the indirect "emancipation" of the medulla is perhaps analogous to the cortex-inhibiting function of medullarin −, although the experiments herein described cannot be interpreted as showing an identity of testosterone propionate and medullarin −. Previous studies on hormonal sex reversal as well as parabiosis suggest instead that the sex hormones exert an influence upon the inductors rather than directly serving as inductor substances.

SUMMARY

Complete sex reversal of large Rana clamitans larvae from females to males with weekly testosterone propionate injections is obtained in 95 days with a total of 4 mg. (at .25 mg./wk.), in 123 days with a total of .9 mg. (at .05 mg./wk.), and in 144 days with a total of .525 mg. (at .025 mg./wk.). When the .25 mg. dose is administered alternately weekly and biweekly, sex reversal occurs in 95 days, although the total amount of hormone used is reduced to only 2 mg. It appears that the minimum time for sex reversal is not much below 95 days. Three sex-reversed males remained alive long enough to produce sperms. Addition of gonadotropins, for concentrations and periods used, had no influence on degree or rate of change in animals receiving sex hormone. Estrogens produce only slight changes, causing some ovocyte degeneration in females and distention of the proximal part of the rete apparatus in males.

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NOTES AND COMMENTS

SEASONAL VARIATION OF RABBITS TO PITUITARY EXTRACTS

In 1929, Bellerby showed that ovulation in the unmated estrus rabbit could be produced by the administration of anterior pituitary extracts. In the same year, Friedman (1929) produced the same effect by the injection of extract of urine of pregnancy. Hill, Parkes, and White (1934) demonstrated that the rabbit ovulation test could be put on a quantitative basis with reasonable accuracy by using a large number of animals for assaying. Bradbury (1944) also noted an individual variation in the sensitivity of rabbits to pituitary extracts. Although he did not carry his experiment through for an entire year, he saw that there was a seasonal trend in sensitivity. Hill, Parkes, and White (1934) treated groups of rabbits at regular intervals and concluded that the rabbits become less responsive to a gonadotropic substance after the third injection. They did not take into consideration that the decreased responsiveness might be due to a seasonal decrease in sensitivity.

In using the rabbit ovulation test for assaying sow, sheep, male and female rabbit pituitary powders, we find that there is an individual variation as well as a seasonal variation.

METHOD

Rabbits, which had been separated for 30 days or longer, were the animals used in testing the potency of the acetone dehydrated pituitary powders obtained from sow, sheep, male and female rabbits. The whole gland was used. The pituitaries from a large number of male and female rabbits were collected in our laboratory immediately after death. The glands were dropped into jars of acetone, there being a separate jar for each sex. Later the glands were ground up in a mortar to a fine powder. A few milligrams of the various powders were weighed out and then placed into flasks. Distilled water was added so that 1 cc. of the suspension was equivalent to 1 mg. of powder. The least amount of the aqueous solution, which after being injected into the marginal ear vein of a rabbit caused the rupturing of the graafian follicles within 16–18 hours, was taken as the unit value for the powder. The solution was carefully shaken before injections were made. Sterile laparotomies were performed under ether anesthesia.

RESULTS

The results obtained for the various pituitary extracts during the 12 months from January, 1944 to January, 1945, show that there is a definite seasonal variation in sensitivity.

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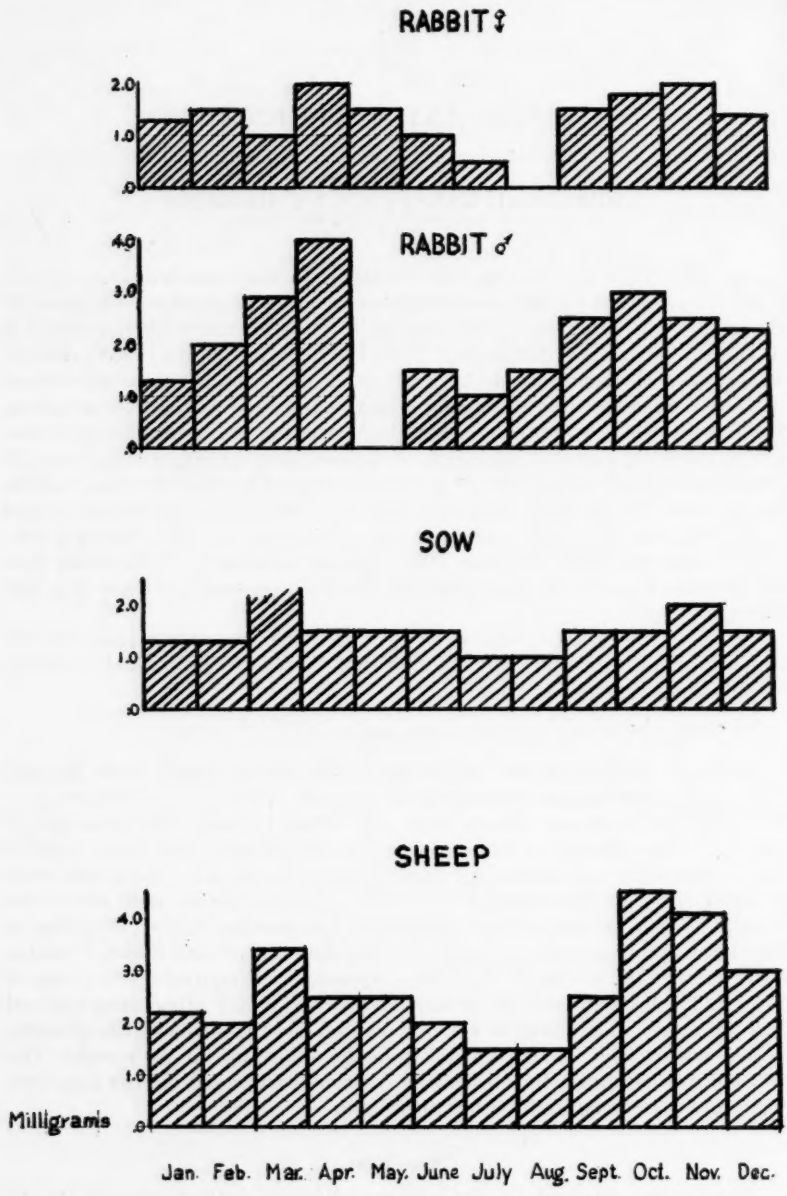
RABBIT ♀
2.0
1.0
.0
RABBIT ♂
4.0
3.0
2.0
1.0
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SOW
2.0
1.0
.0
SHEEP
4.0
3.0
2.0
1.0
.0
Milligrams
Jan. Feb. Mar. Apr. May. June July Aug. Sept. Oct. Nov. Dec.

FIG. 1

The most striking observation is the increased sensitivity of the rabbits to all extracts assayed during July and August and January and February. A marked decrease of potency occurred during September, October, and November; again in March and April.

There is also a definite quantitative difference in the gonadotropic potency of the extracts from the sow, sheep, male and female rabbit pituitary glands. The sow pituitary extract has more gonadotropic activity than the sheep; the female rabbit exhibits more than the male rabbit.

DISCUSSION AND CONCLUSION

Apparently there is a definite seasonal variation in the sensitivity of rabbits to pituitary extracts. This is in accord with the assumption of Bradbury (1944). Variation in the sensitivity of the rabbit to the various pituitary powders is not influenced by the body weight of the animal nor by the number of previous results obtained from the same animal. Hill (1934) found that the gonadotropic potency of the female rabbit pituitary is stronger than the male. Our results are in agreement. Also, the gonadotropic potency of the sow pituitary is found to be stronger than the sheep. Friedman and Friedman (1939) considered light as a possible environmental factor, since temperature and diet had been kept constant. It was only when the animals were exposed for 66 days to the sunlamp or 95 days to electric lamps that a substantial increase in the hormonal content was obtained. The only explanation given seems to be some inherent rhythm in the animal. From our result there seems to be an inherent rhythm of the animal which is diurnal and seasonal.

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NEW BOOK

ANNUAL REVIEW OF BIOCHEMISTRY. Vol. XIV. James Murray Luck, Editor. $5.00. x+856 pp. Stanford University Press, Stanford University, California, 1945.

The fourteenth Annual Review of Biochemistry has continued the tradition established in earlier years by presenting informative summaries of the year's accomplishments. The volume is the largest of the series. In the words of the Editors, this is due to the fact ". . . that there were no withdrawals from authorship, and that several of the reviews which were unexpectedly long did not permit of appreciable curtailment in the editorial office." Although the reviewers this year, as in past years, have labored under " . . . unremitting pressure . . . and the continued difficulty of gaining access to much of the literature deserving of review," the large number and size of the reviews may perhaps indicate that the turning point has been reached and that the demands upon scientist's time may be lessening. However, the concurrent development of the atomic bomb, as well as the other achievements of science during the war, has placed new and heavy responsibilities on the shoulders of investigators.

The principal endocrine chapters in the volume, following past custom, concern the chemistry of the steroids by W. L. Ruigh, carbohydrate metabolism by J. A. Russell and the chemistry of the hormones by W. T. Salter. In addition, the endocrinologist will find information of interest in the chapters concerned with enzymes, proteins, nucleoproteins and phosphorus-containing compounds.

EDWARD W. DEMPSEY